NOVEL SHORT-TERM TESTS FOR ENVIRONMENTAL CARCINOGENS

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LIST OF ABBREVIATIONS

ALV	Avian leukosis virus
AMV	Avian myeloblastosis virus
Ap	Ampicillin
API	Activator protein 1
bp	Base pairs
BL	Burkitt's Lymphoma
BS	Blooms syndrome
cAMP	Adenosine-3',5'-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
CH	Cumene hydroperoxide
CML	Chronic myelocytic leukaemia
cGMP	Guanosine-3',5'-cyclic monophosphate
dbcAMP	N^2,$O^{2'}$-dibutyryl adenosine-3',5'-cyclic monophosphoric acid
dbcGMP	N^2,$O^{2'}$-dibutyryl guanosine-3',5'-cyclic monophosphoric acid
DDT	Dichloro 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane
DEL	Deletion (intrachromosomal recombination)
DEN	Diethyl nitrosamine
DES	Diethyl stilbesterol

DM's	Double minute chromosomes
DNA	Deoxyribonucleic acid
DPN	Dipropyl nitrosamine
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra acetic acid
EGTA	Ethyleneglycol-bis-(ß-amino-ethylether) N,N'-tetra acetic acid
EMS	Ethyl methane sulphonate
ENNG	N-ethyl-N-nitro-N-nitrosoguanidine
ENU	Ethyl nitrosourea
FB	Foldback (element)
FeLV	Feline leukaemia virus
GDP	Guanosine diphosphate
Gm	Gentamycin
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HTLV	Human T-cell leukaemia virus
ICR	Interchromosomal recombination
IHF	Integration host factor
Kb	Kilobases
Km	Kanamycin
LINES	Long interspersed repeated sequences
LT	Long T antigen
LTR	Long terminal repeats
MC	Mitomycin C
MMLV	Murine Maloney leukaemia virus

MMS	Methyl methanesulphonate
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
MNTS	N-methyl-N-nitroso-p-toluene sulphonamide
MNU	Methyl nitrosourea
mRNA	Messenger ribonucleic acid
Nal	Nalidixic acid
NF	Neurofibromatosis
NM	Nitrogen mustard
N-mbcAMP	N^2-monobutyryl adenosine-3',5'-cyclic monophosphoric acid
N-mbcGMP	N^2-monobutyryl guanosine-3',5'-cyclic monophosphoric acid
NNG	Nitro-nitrosoguanidine
O-mbcGMP	O-monobutyryl guanosine-3',5'-cyclic monophosphoric acid
o/n	Overnight
PH	Phenyl hydrazine
(Ph')	Philadelphia chromosome
PNNG	N-propyl-N-nitro-N-nitrosoguanidine
PQ	Paraquat (methyl viologen)
RB	Retinoblastoma
RNA	Ribonucleic acid
SA	Splice acceptor
SCE	Sister chromatid exchange
SD	Splice donor
SD	Standard deviation (σ n-1)

SINES	Short interspersed repeated sequences
SNP	sodium nitroprusside
SV40	Simian virus 40
Tc	Tetracycline
TEA	Triethanolamine
Tf	Transposition frequency
TMU	Tetramethyl urea
tRNA	Transfer ribonucleic acid
VHL	Von Hippel Lindau syndrome
X	Xanthine
XP	Xeroderma pigmentosum

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This thesis is dedicated to the memory of FRANK LESTER ASTBURY

(1919-1990)

SUMMARY

1. An hypothesised role for cGMP in the regulation of bacterial transposition (Wilkins and Swoboda, 1987) was investigated. No effects on transposition, in either of the two existing transposition tester strains, by membrane permeable derivatives of cyclic nucleotides, or other known modifiers of cyclic nucleotide levels in other systems, was observed, with the exception of a large enhancement of transposition frequency (tf) by the mutagen MNNG.

2. Relatively small increases in tf induced by the oxidative mutagens paraquat and mitomycin C, indicated only mild transposogenic effects by oxygen free radicals, in contrast to their known clastogenic activities.

3. Two new transposition assays were developed. The first based on the indirect measurement of Tn*5* transposition into a self-transmissible plasmid in *E. coli* K12 strains, gave accurate and easily quantifiable measurements of tf, with the potential of facile adaption to the study of other transposons. This system was used to investigate the effects of *recA* and *dam*, *dcm* mutations. *dam*, *dcm* mutants show elevated levels of baseline tf but no change in the sensitivity towards the transposogenic effects of MNNG. *recA* hosts were hyper sensitive to MNNG toxicity, but not to transposogenicity.

The second new system, an assay of Mud1(Apr, *lac*) transposition in strains of *S. typhimurium* developed for use in the Ames test, showed that mutagenic and transposogenic effects of MNNG are separate. The use of a transposition based assay for mutagenicity/carcinogenicity seems unreasonable.

4. A test for mutagenic and non mutagenic carcinogens based on the induction of inter- and intrachromosomal recombination (Schiestl, 1989) was investigated. The test detected paraquat, mitomycin C and chemical oxidants as recombinogenic. Similarities in dose response profiles of oxidative mutagens and non-mutagenic carcinogens suggest a possible role for free radical species in the carcinogenetic effect of some non-mutagenic carcinogens.

CHAPTER 1: INTRODUCTION.

1.1 Origins of Human Cancer.

1.1.1 Epidemiology.

In the modern industrialised world, the major killers of the past, famine and widespread disease, have largely been eradicated. With the resulting rise in life expectancy over the last 150 years from roughly 30 to 70 years, cancer has emerged as a major cause of death. Next to cardiovascular disease, cancer is the largest cause of mortality, accounting for 17% of the total deaths in the United States in 1968 (Cairns, 1978).

It is widely accepted that cancer is largely avoidable. It has been suggested that in the U.K. alone, it is possible to reduce the age-specific risks in middle to old age by 75%, through changes in personal behaviour, adequate control of hormonal balance and prevention of viral infection (Doll, 1987). An obvious example is lung cancer, now the most common cancer in the UK and many other western countries. It was comparatively rare until about 1930. From then on, a steady and dramatic rise in incidence among males was observed up to 1970. This correlated directly, allowing for a 20 year gap between commencement of smoking and the onset of cancer, with the increase in cigarette smoking.

There are more than 100 different types of cancer. Epidemiological studies on the world-wide variations in incidence provide evidence for their avoidability.

In almost all populations 4-5 types of cancer account for 50% of the total cancer incidence. These 4-5 differ from population to population, thus none is inevitable (Berg, 1977). All cancers common enough to cause greater than 1% of deaths by the age 75, in either males or females of a given

population, vary in incidence in different geographical regions by at least four fold, and often much more. Skin cancer affects more than 20% of people in Queensland, Australia by age 75, yet has a 200 fold lower, incidence in Bombay, India (Doll, 1977).

Whereas the cause of this huge variation is clearly the exposure of mainly European skin types to very strong sunlight (and hence UV irradiation) in Australia, other cancers which are common in certain populations have not yet been associated with *specific* causal factors. Cancers of the oesophagus, stomach, bowel and breast have world-wide variations of 100, 12, 40, and 8 fold respectively. That these cancers are due to environmental factors can be inferred from the fact that oesophageal cancer occurs in highly localised epidemics and that for the other types of cancer, the incidence in a population can vary from generation to generation, or upon migration of that population to an area with a different level of risk. In a 1965 study it was shown that Japanese men living in Japan, had a death rate from stomach cancer 6.5 greater than their caucasian, Californian counterparts. Japanese immigrants to California however, had only a 4.6 fold higher death rate, and this was reduced in their sons to 3 fold. Conversely, the death rate from cancers of the colon and prostate rose in first generation immigrants, from 1/5 and 1/10 the rate respectively of native Californians, to $^4/_5$ and $^1/_2$ the rate. In the sons of Japanese immigrants, the rates of these cancers rose to $^9/_{10}$ and 1 times the rate respectively (Buel and Durin, 1965).

Apart from the obvious links of skin cancer with sunlight, and lung cancer with smoking, identification of specific risk factors has often proved very difficult. In the case of oesophageal cancer it would seem that

different environmental risks may apply to different high incidence areas. In the U.S. the cancer amongst whites is associated with a combination of cigarette smoking and high alcohol consumption, though neither factor alone carries much increased risk (Berg, 1977). A preliminary study in France showed that the risk was 45 times greater in males who smoked in excess of 20 cigarettes, and drank 81g of alcohol (about 7 whiskies) per day, than in those who smoked less than 10 cigarettes or drank less than 40g of alcohol (Doll, 1977). In Sweden, the primary cause of oesophageal cancer is dietary deficiency associated with Plummer Vinson Syndrome and epidemics in the Caspian and Transkei also show more relation to dietary deficiency than to exposure to any potent carcinogen. There is also a high incidence of the disease in Iran which cannot be explained by alcohol and tobacco consumption.

One suggested link in these cases is the possible exposure of the susceptible, pre-mitotic cells of the oesophagus to ingested carcinogens, by a breach in the usually protective layer of post-mitotic squamous cells. Alcohol is known to increase the permeability of this layer, as is hot tea, which has also been linked to an increased risk. Other types of oesophageal injury, such as those caused by Chaga's disease, achalasia or Lye strictures, also increase the risk of developing the disease, although it is not known whether dietary deficiencies can lead to a breakdown in the integrity of this protective layer. Pathological studies have shown that early stages in the development of colonic and gastric cancers do show structural changes involving the movement of pre-mitotic cells to the exposed mucosal surface (Berg, 1977).

Generally, poor and rich populations would seem to be at risk from different types of cancer (the main exception being lung cancer caused

by smoking). In the case of cancers of the alimentary tract, the poorest populations are at greater risk of developing oral, pharyngeal, oesophageal and hepatic cancers. As the wealth of a population increases and the average calorific intake is boosted by a higher starch consumption, so the risk of stomach cancer is seen to increase. Richer still populations show a decrease in stomach cancer and an increased risk from colonic cancer, associated with the replacement of dietary starch with animal fat and protein. Richer populations also tend to have higher levels of breast, prostate and endometrial cancers suggesting a possible dietary link with these cancers. In contrast, cancer of the cervix uteri is common in poorer areas where the level of hygiene is low (*ibid*).

Such broad-based epidemiological studies can highlight certain environmental influences on the rate of cancer incidence, although they fail to reveal *specific* causative factors (and hence possible remedial measures). It is very difficult to pinpoint specific differences in lifestyle between populations of widely differing cancer profiles, and the identification of causative factors is hampered by the complex nature of cancer induction.

That the development of a cancer can be a multi step process has been recognised since the early promoter/initiator experiments (Berenblum and Shubick, 1947), and it is now recognised that several factors, some of which may not be carcinogenic *per se* (as in the case of alcohol with oesophageal cancer), may interact to form cancer in man. Viruses may also be co-carcinogens in man; in areas of Africa and S.E.Asia, where there is a high incidence of liver cancer, due to the ingestion of aflatoxin B1 from tropical moulds, the risk of contracting the disease is increased if the individual is infected with the Hepatitis B virus (Searle, 1986).

In areas where Burkitt's Lymphoma and cancer of the

nasopharynx are common, they are closely associated with the Epstein Barr virus (EBV) (Doll, 1977). This virus can be used to immortalize *in vitro* cultures of human B lymphocytes, and causes malignant lymphoma when injected into S. American Cottontop marmosets. There is also strong evidence to suggest that cancer of the cervix can be caused by a venereally transmitted virus (Berg, 1977) (the role of viral oncogenes in carcinogenesis is discussed in detail later).

Where specific causative agents for a given type of cancer have been identified, it has often been through the unwitting exposure of certain groups of people to large doses of the agent, either through industrial procedures, or sometimes their use as therapeutic drugs. About 20 chemicals have been shown in this way to cause cancer in humans (though several thousand have been identified as carcinogenic in laboratory experiments).

The first such observation was made as long ago as 1775 by Pott, who reported the extremely high incidence of cancer of the scrotum among young chimney sweeps of the time, who came into contact with coal soot from being "..thrust up narrow, and sometimes hot chimneys, where they are bruised burned and almost suffocated;".

Next to radioactive materials, probably most data is available for the risk of lung cancer from the inhalation of arsenic trioxide by copper smelters and pesticide workers, who were exposed to high concentrations in air (>100 $\mu g\ m^{-3}$) before the risk was identified. Asbestos has also been shown to cause lung cancer and mesothelioma in workers in asbestos factories and other asbestos polluted work environments, such as shipyards. Several studies show a linear relationship (allowing for age) between cumulative exposure to certain asbestos fibres, and risk of death from cancer (Doll, 1987).

Much attention has also been paid to the links between lung cancer and the combustion products of fossil fuels. Many chemicals in the fumes from burning coal and oil, have been shown to be carcinogenic to laboratory animals, though the carcinogenic potential of such fumes is generally seen to be proportional to the concentration of benzo(α)pyrene (BP), and this is thus used as an index of the carcinogenic potential of polluted air. Workers in the coal gas industry were regularly exposed to levels of atmospheric BP of about 30μg m^{-3}, and experienced an 80% increase in the risk of developing lung cancer. Atmospheric pollution from combustion products, has often been blamed for the slight increase in the incidence of lung cancer amongst residents of urban areas, compared to those living in the country. However, atmospheric levels of BP in towns are about 10,000 times less than those experienced in industry (though levels in the past were up to 40 times higher), and it would appear unlikely that they could represent a major cause of urban lung cancer.

Similarly, where other agents have been identified as carcinogens, steps have generally been taken to minimise their exposure to humans, and where large populations have inadvertently been exposed, the concentrations of the agent have been much smaller than those experienced by the group in whom the increased risk was observed. It has been estimated that current levels of arsenic and asbestos pollution in urban areas may account for about 0.1% each of all lung cancers in these areas, and that the risk from a lifetimes exposure to city levels of BP is c. 1×10^{-4} (*ibid*).

Although exposure of such large numbers of people to even very low levels of carcinogens in the environment is almost certainly

responsible for a large numbers of cancers and the continued identification and containment of potential carcinogens is vital to avoid future tragedies. current levels of environmental pollution cannot reasonably be blamed for more than a small percentage of avoidable cancers worldwide. In fact, apart from the increase in lung cancer due to smoking, there is no real evidence to suggest that the general incidence of cancer has increased at all in recent years. Aspects of long-term lifestyle and culture must therefore, contain as yet undiscovered risk factors.

Much epidemiological evidence points towards diet as a major factor in many types of cancer. The human diet contains a large variety of chemicals recognised as mutagens and carcinogens in laboratory tests (Ames, 1982).

Safrole, estragole and several of their metabolites, which have been proved carcinogenic in rats, are present in many edible plant species, notably sassafras (75% safrole) and black pepper which contains safrole and about 10% by weight of a closely related piperine. Black pepper extracts have been shown to cause cancer in mice at a variety of locations, at a dose of $4mg^{-1}kg^{-1}day^{-1}$. estimated human intake is up to $2mg^{-1}kg^{-1}day^{-1}$.

Mushrooms contain large amounts of hydrazines. Most hydrazines which have been tested are carcinogenic. Pyrrolizidine alkaloids, which are carcinogenic, mutagenic and teratogenic, are present in thousands of plant species, especially herbs and herbal teas (often >1% by weight).

Several chemicals isolated from heated protein and several pyrolysed amino acids have been shown to be carcinogens in rodents (Sigimura and Sato, 1983). The amount of burnt material ingested by westerners may be several grams per day. Coffee also contains a large amount

of burnt material, along with about 250mg cup^{-1} of the mutagen chlorogenic acid, and 100mg cup^{-1} of caffeine which inhibits a DNA-repair system, and can increase tumour yields experimentally (Arumuth and Berenblum, 1981).

A high intake of animal fat has often been associated with an increased risk of colon cancer and breast cancer observed in western industrialised societies, one theory is that a high amount of fat, and low dietary fibre intake, may alter the composition of the intestinal flora, favouring anaerobic bacteria such as *Clostridium paraputrificum*, which can produce carcinogens *in situ* by the dehydrogenation of bile acids (Doll, 1977).

Several human cancers such as cancer of the stomach and oesophagus, may be associated with the formation of nitrosamines and other carcinogenic nitroso compounds from nitrates ingested through the consumption of vegetables, or through the drinking of polluted water. Evidence however is often contradictory, as a high intake of nitrate does not necessarily mean a high risk of contracting cancer. People working in the U.K. nitrate fertiliser industry, who are exposed to very high levels of nitrates do not show any increased risk of cancer, and it may be that nitrate only poses a risk when normal diet is deficient in vitamin C, which at physiological amounts is an efficient inhibitor of N-nitrosation, lack of fresh fruit and vegetables in the diet is often associated with gastric cancer (Doll, 1987).

Overall, it can be seen that cancer is a highly complex disease with many possible causes. Many agents present in our environment, whether naturally or through pollution, may put us at an increased risk of developing cancer, either directly, or via interaction with other factors such as diet or the compromising of protective mechanisms. Thus, in trying to identify possible methods for reducing our cancer risks, it is necessary not only to identify

potential carcinogens, but to investigate how they interact with the body on a gross level (ie. what factors can affect the bio-availability of the agent to the tissues susceptible to the their actions), on a metabolic level (do certain agents become more dangerous through *in vivo* enzymatic action, or can additional factors affect the efficiency of protective metabolism), and on a molecular level (how does the ultimate carcinogen interact with a target cell, to cause a switch to a de-differentiated state with uncontrolled replication?).

1.1.2 The Genetic Basis of Cancer Development.

The evidence that cancer is a genetic disease, arising from damage to cellular DNA, comes from a wide range of observations. The carcinogenic potential of many chemicals is closely related to their potency as mutagens (McCann and Ames, 1976; Ames, 1979); there are several recognised hereditary predispositions to cancer (Knudson, 1977); an altered karyotype, arising from chromosomal rearrangements, has been detected in a wide variety of cancers, and often becomes more abnormal as the cancer progresses (Cairns, 1981); and susceptibility to cancer is increased when the ability of cells to repair damaged DNA is impaired (Handwalt and Sarasin, 1986).

Elucidation of the specific genetic changes responsible for cancer development has been greatly aided by the study of animal retroviruses. Retroviruses causing tumours in chickens were amongst the first animal viruses described (Varmus and Brown, 1989). Their ubiquity in vertebrates, unusual biochemistry, ability to perform genetic feats such as transduction and insertional mutagenesis, and pathology (notably in the causation of leukaemias, sarcomas and mammary carcinomas), has lead to a

widespread study of their biology by many investigators over the past 20 years. Studies of retroviruses causing cancer in experimental animals have implicated almost 50 cellular 'oncogenes' in carcinogenesis (Bishop and Varmus, 1985).

The retroviral lifecycle (figure 1.1) begins with the entry of the nucleoprotein core into the target cell, probably via a receptor-mediated endocytosis of the extracellular enveloped particle. The nucleoprotein core consists of 2 copies of the single stranded RNA genome, 7-9 kilo bases (kb) in length, assembled in a 70S complex with a host tRNA used as a primer for DNA synthesis, and sometimes other low molecular weight host RNA's (Coffin, 1985), several virus-encoded proteins including the structural components of the core, enzymes for reverse transcription, proteolysis and proviral integration, and a variety of host components which may be randomly packaged.

Inside the host cell, a double stranded DNA copy of the viral RNA (which has the same polarity as mRNA) is synthesised by reverse transcription. The DNA-protein complex then migrates into the nucleus, where some of the DNA is circularised and integration of the proviral DNA into the host cell genome occurs at any of a wide variety of sites. Integration of the provirus results in the loss of 2 base pairs (bp) from each long terminal repeat (LTR) of the unintegrated provirus (the majority of unintegrated viral DNA molecules isolated have 3' ends recessed by 2bp relative to their 5' ends, probably representing a late intermediate in the integration pathway), and the generation of short stretches of duplicated host DNA (4-6 bp) at the integration site, forming a direct repeat flanking the proviral DNA (Varmus and Brown, 1989) as seen for most transposable elements (see later section).

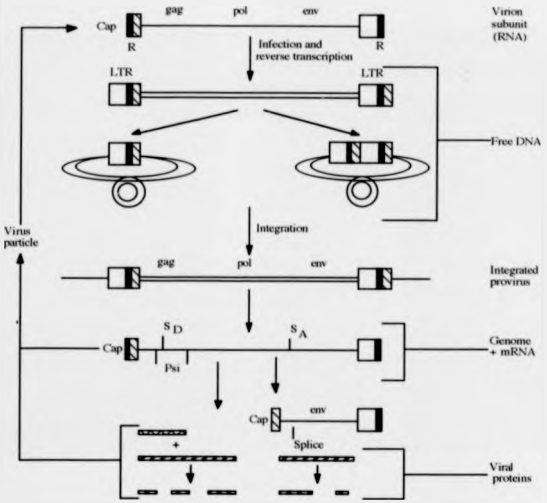


Figure 1.1. Schematic representation of genetic events in the retroviral lifecycle. The structure of the immediate proviral precurser is ambiguous (Varmus and Brown, 1989). R: inverted repeat; LTR: long terminal repeat; *gag*, *pol*, *env*: genes encoding viral proteins; *psi*: region associated with packaging of viral RNA; Cap: 5' 7-methyl guanine modification; S_D: splice donor; S_A: splice acceptor.

Retroviruses are related in both structure (typified by the presence of LTR's a few hundred bp's in length) and function ('transposing' via RNA intermediates converted to DNA by reverse transcriptases) to the

family of insertion elements known as retrotransposons, which include the Ty elements of *Saccharomyces cerevisiae* and the *copia*-like elements of *Drosophila*. The infection of a new target cell, beginning with a provirus integrated into the genome of its host, is, in effect, an intercellular transposition event.

Once integrated into the host genome, the proviral DNA can contribute to the development of a neoplastic phenotype by one of 3 postulated mechanisms.

(1) Insertional Mutagenesis: Avian Leucosis Virus (ALV) DNA contains promoter sequences in its LTR's. Normal RNA synthesis is initiated from the left LTR, however the same sequence in the right LTR could initiate synthesis in an adjacent cellular proto-oncogene (c-*onc*) (figure 1.2). Hayward, Neel, and Astrin (1981) isolated RNA from normal and ALV-induced lymphoma tissues, and using cDNA probes for the oncogenes *src*, *fps*, *myb*, and *myc*, found that levels of c-*myc* mRNA were 30-100 fold higher in lymphoma tissues than in normal or infected but non-neoplastic cells. They also found that c-*myc*-specific cDNA bound to the same mRNA as cDNA from the 5' end of the virus, showing that c-*myc* mRNA had viral sequences corresponding to about 100 bp of the 5' terminal. Analysis of lymphoma DNA's showed that 31 out of 37 samples had proviral information inserted adjacent to c-*myc*, suggesting that the enhanced c-*myc* expression was due to promotion from the proviral DNA. The c-*myc* gene may also be activated by insertion of retroviral DNA within, or occasionally downstream of the gene (Noori-Daloii *et al*, 1981; Payne *et al*, 1982).

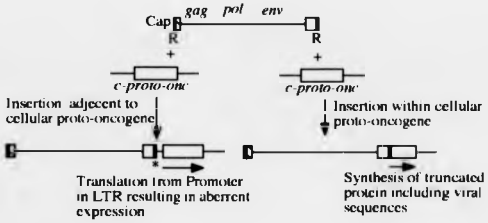


Figure 1.2. Diagrammatic representation of retroviral insertional mutation of a cellular proto-oncogene. R: inverted repeat; LTR: long terminal repeat; *gag*, *pol*, *env*: genes encoding viral proteins; Cap: 5' 7-methyl guanine.

Murine Maloney Leukaemia Virus (MMLV) also causes transcriptional activation in murine thymoma cell lines (Adler *et al*, 1988). Levels of the protein $p56^{lck}$ were found to be elevated in 2 cell lines when MMLV proviral DNA was inserted between the two usual c-*lck* promoters. In these cells $p56^{lck}$ was encoded by a virus-*lck* hybrid containing the 5' untranslated region of MMLV.

Insertional mutagenesis can also occur when proviral DNA inserts within a cellular proto-oncogene, resulting in the expression of an altered gene product (figure 1.2).

The induction of erythroleukaemia by AVL in chickens begins with insertion of the provirus into the cellular gene c-*erbB*, resulting in the expression of a gene product with an amino-terminal truncation which is apparently responsible for the transforming activity (Bishop, 1987).

MMLV also causes insertional mutation of the cellular proto-

oncogene c-*myb* in myeloid leukaemia cells, by truncating the gene at either the 3' or the 5' ends (Lavu and Reddy, 1986; Shen-Ong *et al*, 1986) (interestingly, a combination of both the 3' and 5' truncation of c-*myb* appears responsible for the high transforming activity of the viral oncogene v-*myb* in Avian Myeloblastosis Virus (AMV) (Klempnauer *et al*, 1983)).

(2) Oncogenic Transduction: During their evolution, many animal retroviruses have picked up cellular proto-oncogenes which become part of the viral genome and are expressed along with viral genes. During the transductional process, the cellular proto-oncogenes obtain transforming activity by coming under the control of strong viral promoters (which the target cells may not be able to control), the accumulation of point mutations, deletions and genetic substitutions (which may alter the biochemical activities of the gene products by the removal of allosteric controls) or by constitutive activation or the loss of self regulatory functions (Bishop, 1987).

The transduction of cellular proto-oncogenes into retroviruses is generally a very rare event, and thus it has not proved possible to determine the mechanism experimentally. However, several observations, such as the lack of introns in the v-*onc* sequences (suggesting RNA splicing as part of the process), and the very rare transduction of non-oncogenic genes (suggesting the preliminary step of proto-oncogene activation by promoter insertion, followed by neoplastic clonal expansion - increasing the likelihood of transduction), have led to the proposal of a generally accepted model (figure 1.3) (Varmus and Brown, 1989).

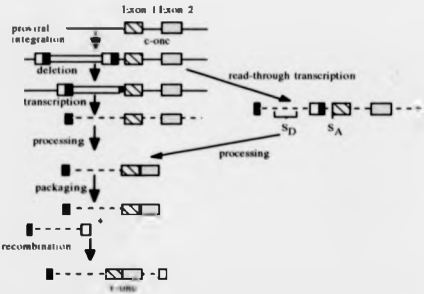


Figure 1.3. Proposed mechanism of cellular proto-oncogene transduction. The provirus integrates into the genome on the 5' side of the *c-onc* gene, in the same transcriptional orientation. Two possible mechanisms may create a virus-host transcript. On the left, a deletion event removes the 3' end of the viral DNA and adjacent cellular DNA (this may or may not include coding sequences), transcription and processing remove *c-onc* introns. On the right, read through transcription from an insertionally mutated provirus 5' LTR creates a hybrid RNA, processing of the transcript creates an RNA similar to to the pathway on the left. After, the hybrid is packaged into a virus particle, provided by an intact provirus in the same cell, along with a subunit of wild-type viral RNA, recombination during reverse transcription creates the oncogenic virus.

The rarity of oncogene transduction by retroviruses, and thus their assumed numerical insignificance in *naturally* occurring tumours, is contrasted by the case of Feline Leukaemia Virus (FeLV), which is widespread in the domestic cat population, where it induces a wide spectrum of diseases. Isolates from cases of FeLV induced multicentric sarcoma have revealed 7 different oncogenes which can confer the tumorigenic phenotype when

carried by the virus (table 1.1) (Neil, 1985). One isolate, GR-FeSV, carries not only the oncogene v-*fgr*, but also a host derived domain related to the actin gene family (Naharro *et al*, 1984). suggesting a possible link between cytoskeletal elements and growth control.

Table 1.1. Transduction of Oncogenes by FeLV.

Oncogene	Associated Cancer
fes	Sarcoma
fms	Sarcoma
abl	Sarcoma
sis	Sarcoma
ki-ras	Sarcoma
fgr	Sarcoma
kit	Sarcoma
myc	T-Cell Leukaemia

In addition to the sarcoma-inducing viral oncogenes, FeLV shows a remarkably high incidence of *myc* transduction, with over 10 reported isolates from feline Leukaemias (Neil, 1985). This has led to speculation about possible homology between FeLV and the c-*myc* locus. Homology between a c-*onc* locus and the 5' recombination site of a provirus inserted upstream in the genome could assist or direct the deletion event thought to be an early step in the transduction process (figure 1.3), and there are several examples to support this (*ibid*).

(3) *Trans*-activation. The group of retroviruses which includes the Human T- cell Leukaemia viruses (HTLV) (figure 1.4), can induce tumours slowly and infrequently. They can transform cultured cells inefficiently though they do not carry any transduced cellular genes, and have not been shown to cause insertional mutagenesis. The *tax* gene of HTLV can however stimulate the production of 2 proteins, interleukin 2 and its receptor, implicated in T-cell growth control. Also, transgenic mice carrying the HTLV *x*-region develop neurofibromas (Varmus and Brown, 1989). The prominent theory for the tumorigenic ability of these viruses is the ability of the *x*-encoded proteins to *trans*-activate certain cellular genes.

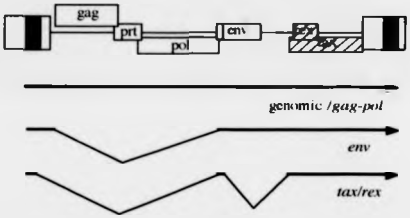


Figure 1.4. Structure of the human retrovirus HTLV II. Boxes gag, pol, etc represent coding regions for their genes; *gag* is assigned to the 0 reading frame, boxes at the level immediately below are in the -1 frame, and those further below in the +1 frame. Spliced and unspliced mRNA's which have been identified are shown below the genome.

The biochemical role played by the products of cellular proto-oncogenes and their oncogenic derivatives is obviously of primary interest in

determining the causes of cancer. To date, only four mechanisms have been identified by which these 50 or so proteins may act: protein phosphorylation by serine or tyrosine kinase activity; metabolic regulation by GTP binding proteins; control of gene expression through control of mRNA synthesis and participation in DNA replication (Bishop, 1987).

The *src* and *ros* proto-oncogenes have been shown to encode related tyrosine kinases, and the transforming potential of their oncogenic alleles is thought to arise through a shift in phosphatidyl inositol metabolism, either directly via their innate lipid kinase activity, or by regulation of other lipid kinases (Weinberg, 1985). It has also been suggested that the submembranous location of the *src* family of proteins indicates a regulatory role in transmembrane processes by modification of proteins such as ion-channel subunits, or by acting directly as subunits for surface receptors (Hunter, 1989). Other oncogene products with tyrosine kinase activity have been shown to be altered growth factor receptors, notably the v-*erbB* gene of avian erythroblastosis virus, which is derived from the chicken epidermal growth factor receptor gene and v-*fms*, which is derived from the gene encoding the receptor for colony stimulating factor-1. Several other oncogene products are assumed, from structural similarities, to be growth factor receptors (*ibid*). Interestingly there is only one oncogene which is known to encode an altered growth factor, *sis*, which encodes an altered form of platelet derived growth factor (Weinberg, 1985).

The role of the serine kinase oncogene products *mos* and *raf* is less clear, though they show structural and functional similarities to protein kinase C, and may also be involved in growth factor signalling mechanisms (Grunicke, 1990; Hunter, 1989).

There is now a great deal of evidence suggesting that the ras gene family (Harvey, Kirsten, and N-*ras*), encodes GTP-binding proteins (G-proteins) involved in signal transduction from cell-surface receptors, possibly growth factor receptors (Bos, 1988). The $p21^{ras}$ product of the *ras* proto-oncogene shows remarkable homology to known G-proteins in both amino acid sequence and function, binding both GTP and GDP, and showing intrinsic GTP-ase activity. Several growth factors act via a phosphatidyl inositol bisphosphate-specific phospholipase C, which is under the control of a G-protein, and *ras*-transformed cells have been shown to exhibit elevated levels of inositol polyphosphate turnover. In addition, injection of anti-*ras* antibodies inhibits the mitogenic activity of serum growth factors (Grunicke, 1990). Thus, an activated *ras* oncogene could encode a constitutively activated G-protein, triggering a mitogenic pathway usually mediated via growth factor binding to cell surface receptors.

It is also highly tempting to assign a role in the growth factor signal mechanism to those oncogenes whose products act within the cellular nucleus. In fact, it is known that growth factors strongly stimulate expression of the nuclear proto-oncogenes c-*myc*, c-*fos*, and *p53* (Weinberg, 1985).

The biochemical functions of these proteins is, as yet, poorly understood, although the available evidence so far points towards their action as *trans*-acting regulators of gene expression (Eisenman, 1989).The most widely studied nuclear proto-oncogenes are those of the myc family, rearrangements of the c-*myc* locus have been implicated in a wide range of neoplasms in many species. Functions associated with c-*myc* include regulation of DNA synthesis (Studzinski *et al*, 1986), regulation of post-transcriptional events and involvement in RNA processing (Eisenman, 1989).

The *fos* nuclear oncogene has recently been shown to encode a component of a nucleoprotein complex which specifically associates with gene regulatory sequences. The second component of this complex, the c-*jun* gene product, is also a proto-oncogene product which is probably identical to the mammalian transcription factor, activator protein-1 (AP-1). Fos-jun complexes bind with high affinity to AP-1 binding motifs in various cellular genes which, interestingly, include the c-*myc* gene. Both c-*fos* and c-*jun* are rapidly activated by a wide range of growth factors and/or differentiation-promoting agents, and are highly modified by phosphorylation, again suggesting a link with growth factor signals (*ibid*).

Another class of oncogenes is that carried by tumour inducing DNA viruses, papovaviruses (polyoma viruses, SV40 and papilloma viruses), the adenoviruses and the herpes viruses (notably Epstein- Barr virus (EBV)). The oncogenes carried by these viruses are not derived from cellular genes, but exert their transforming activity through their action as *trans*-activators of cellular functions. Examples of such *trans*-activators are the mT protein of polyoma virus, which has been shown to activate the c-*src* gene product $pp60^{src}$ by binding directly to the protein (Eckhart, 1989), the large T (LT) antigen of SV40, which may act by binding to, and inactivating the cellular p53 protein which can *suppress* transformation (*ibid*), the E1A gene product of adenovirus, which is a trans-activator of cellular transcription (Shenk, 1989) and the EBNA-2 protein of EBV which, in infected ß-cells, can stimulate DNA synthesis, activate high level expression of the ß-cell activation marker CD23 and may also stimulate expression of ß-cell autocrine growth factors (Keiff and Leibowitz, 1989).

In recent years another class of cellular genes has emerged as

being of importance in the development of cancers. In this case it is the *loss* of a gene function which contributes to the development of the neoplastic phenotype, these genes are therefore referred to as anti-oncogenes or tumour suppressor genes. There is a wide body of evidence supporting the existence of such suppressing functions in tumours of the fish *Xiphophorus*, the fruit fly *Drosophila*, and mice (for a review see Schwab, 1989).

In humans, the best evidence for tumour suppression comes from the study of the inherited cancer retinoblastoma. Statistical analysis of the inherited and sporadic forms indicated that development of the disease requires two mutational events ('hits'), and that, in the inherited form, one of these mutations is passed on through the germ line. It is now known that these hits occur at the same genetic locus, which has been mapped to the long arm of chromosome 13 (13q14) designated RB1. The high frequency of disease in individuals inheriting one mutated RB1 locus (90%), and the common occurrence of 2 or more separate cancers, reflects the high probability that one of the heterozygous (*rb*/+) retinoblast cells will acquire an inactivating mutation in the second allele. The sporadic form is relatively rare, as any one cell must acquire separate mutations in each copy of the gene in order to form a cancer, and the sporadic disease is invariably monoclonal (*ibid*; Ponder, 1988).

Several groups have identified and cloned a gene which appears responsible for retinoblastoma suppression. This RB1 gene is either deleted or aberrantly expressed in 20-40% of retinoblastomas examined. The RB1 gene is large and complex (200kb, 27 exons), and small mutations such as single base pair substitutions, which may result in the formation of a functionally defective protein, are very difficult to detect. The present available evidence

suggests that mutations of both RB1 alleles may be involved in all retinoblastomas (Schwab, 1989). Retinoblastoma patients also have a high incidence of secondary tumours, notably osteosarcomas, at a frequency of about 25%. The neoplastic phenotype of both retinoblastoma and osteosarcoma cells can be suppressed *in vitro* by the induction of an RB1 expression vector. In addition, genetic alterations in the RB1 gene have been implicated in other tumours such as mammary and small cell lung cancers (*ibid*).

The RB1 gene product, p105-RB1, is a 105kd phosphoprotein, which is widely expressed in almost all tissues. It has been shown to associate with various viral oncogenes, notably the E1A protein of adenovirus and the LT antigens of SV40 and polyoma virus. Interaction with these viral proteins may inactivate the suppressing activity of p105-RB1, and raises the question of whether the development of retinoblastoma results from the lack of suppressive binding of p105-RB1 to the product of a *cellular* proto-oncogene. As yet no such evidence is available (*ibid*).

A gene has recently been cloned, which appears to act as a suppressor gene in the formation of Wilms' tumour (a disease accounting for 85% of childhood kidney cancers). The gene, which has been mapped to 11q13 on the short arm of chromosome 11, is present in two copies, and is expressed in normal cells, but is missing in some tumour cell lines. The protein produced by the gene has a 'zinc-finger' region, characteristic of DNA-binding transcription factors which can enhance or suppress gene expression (Hoffman, 1989).

Table 1.2. Loci of alleles associated with inherited predispositions to cancer.

Inherited syndrome	Associated loci
Retinoblastoma	13q
Wilms' tumour	11p
Von Hippel Lindau (VHL)	3p
Polyposis of the Colon (familial)	5q
Multiple Endocrine Neoplasia 1 (MEN1)	11q
MEN2	10
Neurofibromatosis (NF) type 1	17
type 2	22

(q, long arm; p, short arm)

Loss of the second allele in the tumour cells of inherited cancers is often visible cytogenetically, as the loss of a small piece, or all of the chromosome. In this way the loci of inheritable predispositions to other cancers have been located, using Southern blotting techniques, with DNA-sequence polymorphisms as markers for adjacent alleles (Ponder, 1988).

Such double allele losses in the inherited cancers probably indicate the loss of suppressor functions. Sporadic tumours of the same histological types as those associated with VHL, NF2 and other inherited syndromes also show loss of the same loci (*ibid*). However, the explanation may be more complex than this. There may be more than one sequence in the

11p13 region associated with Wilms' tumour, and perhaps abnormalities in other areas of chromosome 11 (Hoffman, 1989). In the case of MEN2, allele losses at the locus associated with inheritability have not yet been found in the corresponding tumours, although loss of alleles on chromosome 1p are common (Ponder, 1988).

Table 1.3. Allele losses associated with common, sporadic cancers.

Tumour	Site of Allele Loss
Bladder	11p
Renal Carcinoma	3p
Lung Small Cell	3p, 13q, 17p
Breast	11p, 13q
Stomach	13q
Colon	5q, 17p, 18q, 22, others
Melanoma	Various

Allele losses are also often seen in many of the common sporadic cancers (table 1.3), although the existence of a corresponding inherited disposition has yet to be shown (*ibid*). Successive deletions, on different chromosomes, are seen during the stepwise progression of a colonic cancer towards metastasis. These deletions probably cause the loss of suppressor genes which would otherwise inhibit different stages in the multistep process. In this case other changes are also required, including the activation of a *ras* oncogene. The successive stages of colonic cancer, and the genetic changes

associated with progression between the steps have been quite well defined over the past few years (Marx, 1989), and give a good insight into the complex nature of carcinogenesis.

1.1.3 Genetic Damage leading to the Development of Cancer in Humans.

Conventional theories of carcinogenesis, derived from experimental observations on the induction of cancers in laboratory animals, have generally described carcinogenesis as a process involving at least two distinct components (Berenblum, 1978). The first, an *initiating* phase, involves the production of a permanent, inheritable change in the DNA sequence of a cell, by direct interaction of the DNA with a conventional mutagen (alkylating agents, intercalating agents, UV irradiation, etc.), followed by a mistake in either DNA duplication during cell replication, or during repair of the lesion. This mutational event is rapid and efficient, often requiring only one small dose of mutagen, and results in a dormant tumour cell.

In the second, *promoting* phase, the latent tumorigenic phenotype becomes expressed, resulting in the growth of a clonal tumour from the previously dormant cell, or one of its progeny. This promoting phase is slow, accounting for the long latent period of many cancers, and was presumed to be epigenetic in origin, associated perhaps with cell surface processes (*ibid*).

This somatic mutation theory of cancer arising from point mutations in cellular genes is however, insufficient to explain the the origins of the majority of human cancers. To date, the only evidence indicating the presence of point mutations in human cancers is for the closely related *ras* family of oncogenes (H-*ras*, K-*ras*, and N-*ras*). *ras* Genes isolated from a wide

variety of human tumours, have obtained transforming activity (ie. the ability to transform the mouse cell line NIH/3T3, into tumour cells) by point mutations in one of 3 codons: codons 12 or 61 (H-*ras*); codons 12, 13 or 61 (K-*ras*) and codons 12 or 13 (N-*ras*) (Bos, 1988). These single base pair mutations result in the substitution of the native amino acid for that codon (glycine at codon 12 and 13, glutamate at codon 61) for one of a number of alternative amino acids.

Analysis of human H-*ras-1* p21 product, and its oncogenic derivative from human EJ/T24 bladder carcinoma cells, where the glycine at position 12 of p21 is replaced by valine, showed that the mutation resulted in a 10-fold lower rate of intrinsic GTP hydrolysis, thus stabilising the active, GTP-bound state of the protein (McCormick, 1989). Other mutated proteins isolated from transformed cells were also invariably found to have reduced GTPase activity, although mutant proteins created *in vitro* can become activated by a reduction in guanine nucleotide binding affinity, resulting in a rapid exchange of bound GDP for activating GTP (*ibid*).

Activated *ras* genes have been found in most types of human neoplasia, although the frequency of incidence varies greatly between tumour types. Mutated *ras* genes are found in up to 40% of colorectal cancers, and 20% of acute myeloid leukaemias, but occur infrequently, if at all, in cervical cancer or chronic myeloid leukaemia (though *amplifications* of *ras* genes were found in 8 of 12 cervical tumours studied) (Bos, 1988). Apparently, mutations of *ras* genes are only one of a number of possible events which may contribute to the development of a given type of cancer. In the case of colonic cancer, where *ras* mutation is common, it is only one of a series of events, including several chromosome losses, which contribute to the progressive development of the malignant phenotype (Marx, 1989).

In his 1981 essay "The origin of human cancer", Cairns questions the importance of such local changes in DNA sequence in the development of human cancer. He points out that if point mutations were a major cause of malignant tumours, then any inherited defect in the cellular mechanisms for the repair of such lesions, would lead to a greatly increased risk of developing common cancers. Patients with the rare inherited disease Xeroderma pigmentosum (XP) have defects in both copies of one of the genes involved in the main excision repair pathway. The result is equivalent to *uvr* mutations of *Escherichia coli*, making the cells of XP patients up to 10 times more sensitive to the mutagenic and lethal effects of most mutagens. However, although the XP defect extends to all tissues of the body which have been tested, there is no apparent increased risk from any of the common, lethal cancers with the major exception of malignant melanoma, caused by exposure of susceptible skin cells to UV radiation from sunlight. Thus, Cairns surmises, the only dangerous point mutagen to which most people are exposed is sunlight, and as death from malignant melanoma is rare in the general population (about 1% of reported cases are fatal), point mutations of the type XP patients are unable to repair, are unlikely to contribute significantly to the incidence of most common, fatal cancers.

In contrast to XP, Cairns highlights the case of another rare, inherited disease, Bloom's Syndrome (BS). BS is an autosomal recessive disorder. BS patients suffer growth retardation, immunodeficiency and importantly, an approximately 100 fold increased risk of death from all cancers (German *et al*, 1984). Cells from BS patients show a high level of chromosome abnormalities, including sister chromatid exchanges (SCE), chromosome breakages, mutations and high recombinogenic activity (German, 1983;

Rünger and Kraemer, 1989; Langlois *et al*, 1989).

The abnormal chromosomal instability of BS cells has been linked to an abnormal oxygen metabolism resulting in high levels of clastogenic oxygen free radicals (Cerrutti, 1982; Kennedy *et al*, 1986), abnormal regulation of Uracil DNA glycosylase (Yamamoto and Fujiwara, 1986), abnormal expression of O^6-methylguanine methlytransferase (Kim *et al*, 1986) and a delayed maturation of newly synthesised DNA (Ockey and Saffhill, 1986). It now seems likely that the primary defect in BS is in fact, an abnormal DNA ligase I, the enzyme responsible for ligation of DNA breaks formed during replication, and repair of nicks and double stranded breaks (including blunt-ended fragments) formed during DNA repair or recombination. Ligase I isolated from 5 cell lines derived from Askenazi BS patients (a group of Jews at a high risk of BS, thought to be descended from a single Polish Jew), showed a slightly reduced molecular weight, a higher heat lability and a 75% reduction in enzyme activity when compared to normal controls (Willis and Lindhal, 1987; Willis *et al*, 1987; Chan *et al*, 1987). A sixth BS line derived from a Canadian Mennonite showed an indistinguishable ligase defect, however a seventh line from an Anglo Saxon sufferer with identical clinical symptoms, had a ligase with normal heat lability, but low activity, which was apparently due to a predisposition to dimerization (Willis *et al*, 1987). Additionally, BS cell lines which had reverted to normal levels of SCE *in vitro*, also showed normal levels of ligase I activity (Willis and Lindhal, 1987). Though limited, this evidence points firmly towards a defect in DNA ligase I as the primary cause of BS. An analogous mutation in *Saccharomyces cerevisiae*, *cdc-9*, resulting in a temperature sensitive DNA ligase, results in a >30 fold increase in recombination, presumably due to the increased

frequency of unrepaired DNA nicks providing single stranded DNA for invasion of a recombinant chromatid (Schiestl and Prakash, 1988).

The high level of cancer risk experienced by BS patients strongly suggests that the kind of chromosome abnormalities seen at a high frequency in cells from these patients are responsible, at least in part, for the acquisition of a neoplastic phenotype.

Abnormal mitoses are a common feature of cancer cells of many types. As early as 1890, von Hansemann suggested that these irregularities were responsible for disordered growth (Chaganti, 1983).

Until recent years, it has been argued that visible chromosomal rearrangements may arise as a result of, rather than the cause of, neoplastic transformations, and that although they are obviously of great importance in the development of cancer, they may only play a role in the later stages, rather than as a direct cause of cancer. Modern genetic techniques however, such as chromosome banding, somatic cell hybridisation and DNA sequence analyses, have highlighted the highly specific nature of chromosome rearrangements associated with various cancers and have also shown the involvement of certain cellular proto-oncogenes in some of these, throwing new light on the possible involvement of such rearrangements in carcinogenesis.

The first and best studied specific rearrangement in a human cancer was reported by Nowell and Hungerford in 1960. The so called Philadelphia chromosome (Ph') of chronic myelocytic leukaemia (CML). Ph' is characterised by a specific translocation involving a reciprocal exchange between chromosomes 9 and 22 (specifically t(9;22)(q34;q11)) resulting in a visibly truncated chromosome 22 (Cairns, 1981; Chaganti, 1983; Groffen *et al*, 1984; Bishop, 1987). The translocation results in a portion of the c-*abl* proto-

oncogene on chromosome 9 moving to a specific locus known as the *bcr* (for "breakpoint cluster region"). The fusion results in a chimeric protein with a higher enzymatic activity and possibly a higher level of expression (Groffen *et al*, 1984; Bishop, 1987). The Ph' chromosome is present early in the sequence of events leading to the leukaemia, and thus may be the primary switch allowing a precancerous stem cell to multiply uncontrolled (Cairns, 1981). In the 10% of CML cases not showing a Ph' chromosome, translocations between 22q and a chromosome other than No.9 may be involved and the prognosis in these cases is often less favourable (*ibid*, Chaganti, 1983).

A second proto-oncogene has been shown to be involved in Burkitt's Lymphoma (BL) and a similar murine cancer. The proto-oncogene *myc*, originally identified as the transforming oncogene of Avian Leucosis Virus, is often found to have moved, in a t(8;14)(q23;q32) translocation, to the precise point in the immunoglobulin heavy chain coding region where breakage and reconnection occur during B-cell development (Marx, 1982). This translocation results in over expression of *myc*, which is unaltered in its regulatory regions (despite 6 nucleotide changes within the coding sequences) and must thus be over expressed due to its position within an active immuno-globulin locus (although this would mean an influence acting over about a 20kb distance (Showe *et al*, 1987)).

Abnormalities of the 11q23 region, including reciprocal translocations, deletions, inverted insertions and homogeneously staining regions (HSR's) have been detected in acute myelomonocytic leukaemias, small lymphocytic cell lymphomas and myeloproliferative syndromes. A human analogue of the viral oncogene v-*ets*, of the Avian erythroblastosis virus E26, named Hu-*ets*-1, has been mapped to 11q23-24. Hu-*ets*-1 and has

been shown to be rearranged and amplified 30-fold in acute myelomonocytic leukaemias showing HSR's at 11q23. The gene is rearranged and amplified 10-fold in a small lymphocytic cell lymphoma with an inverted insertion at 11q23, and moved to chromosome 4 in a t(4;11)(q21;23) translocation of a leukaemic subtype, resulting in altered expression (Sacchi *et al*, 1986; Rogigatti *et al*, 1986). A second v-*ets* analogue, Hu-*ets*-2, is also involved in the t(8;21)(q22;q22) translocation of acute myeloid leukaemia type M2, where its movement from chromosome 21 to 8 also results in altered expression (Sacchi *et al*, 1986). Thus, altered expression of human *ets* oncogenes, resulting from chromosome rearrangements, may be a prime switch in the development of many myeloproliferative disorders.

Amplifications of small regions of DNA containing proto-oncogene sequences, have often been reported as an occasional feature of diverse tumours, and as a recurring specific abnormality of a proto-oncogene in particular types of tumours (Bishop, 1989). Amplifications resulting in greatly increased copy number of specific genes, are characterised by HSR's or by the presence of 'double minute' chromosomes (DM's). Although the mechanism by which these occur is not known, a model for gene amplification involving an initial proto-oncogene duplication, followed by successive recombinations between sister chromatids, has been proposed (figure 1.5) (Pall, 1982).

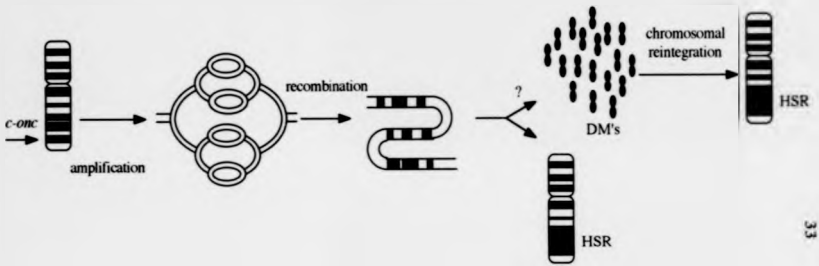


Figure 1.5. Mechanism proposed to explain amplification of oncogenes. The first step is a duplication of the oncogene, successive rounds of recombination result in the appearence of 'homogeneously staining regions' (HSR's) and, via an unknown mechanism, 'double minute' chromosomes (DM's).

Other amplified proto-oncogenes involved in cancers include *erb*-B1 in squamous cell carcinomas, *myc* in breast and lung carcinomas and *ras* in stomach and cervical cancers (Bishop, 1987; Bos, 1988). Amplified proto-oncogenes are generally found however, in cells which are already partially or fully malignant, and their involvement in the initiation of cancer remains uncertain (Bishop, 1989).

The third and most common major chromosome abnormality associated with cancer cells is deletion of parts of, or whole chromosomes. As discussed previously, loss of genetic information may result in loss of anti-oncogenic or tumour suppressor functions. As has been shown, loss of both copies of a suppressor gene leads directly to the development of retinoblastoma. Loss of information on chromosome 5 is thought to trigger the initial step of increased cell growth on the pathway to colorectal cancers (Marx, 1989). The other steps are triggered by further losses on chromosomes 18, 17 and others, while deletions distal to the 3p11 region are consistently observed in renal cell carcinomas and small cell lung carcinomas (Stanbridge and Cavenee, 1989). The inactivation of anti-oncogenes may be equally as important as, and in many cases may supplant the requirement for, the activation of cellular oncogenes in carcinogenesis (Weinberg, 1989).

The apparent paradox between the initiator/promoter model of carcinogenesis, typified by the chemical induction of skin cancers on mouse skin and the observation that cancer in man is more likely to arise from large scale rearrangement of chromosomal DNA is not necessarily as contradictory as it may at first seem. Man has always been exposed to a wide range of mutagens both in his environment and in his food, and thus it is not surprising that he has evolved a range of highly efficient protective mechanisms to

remove these and repair any damage they may cause. It may only be when these mechanisms are compromised as in XP, or overloaded as occurs for example in the chronic exposure of caucasian skin types to strong, tropical sunlight, that a cancer may develop.

The initiator/promoter model acknowledges that the rate determining step in carcinogenesis is the promotion phase. It is now known that the best studied promoting agents, the phorbol esters, which do not bind to DNA and are negative in the Ames mutagenicity assay (ie. they are not point mutagens in the accepted sense), do in fact cause widespread DNA damage. They apparently achieve this by a receptor mediated stimulation of arachidonic acid metabolism, resulting in the production of high levels of intracellular free oxygen radicals (Marx, 1983). Other chemically unrelated compounds, which can generate free radicals, can also act as promoters of mouse skin cancer, and there appears to be a correlation between promoter efficiency and stimulation of the oxidative burst (*ibid*). Additionally, superoxide anion itself (O_2^-), has been shown to act as a promoter of translocation in mouse CH3/10T½/C18 cells initiated by gamma rays or benzo(α)pyrene, mimicking the action of phorbol myristate acetate (PMA) (Zimmerman and Cerrutti, 1984).

Free oxygen radicals damage DNA by causing strand scission, backbone breakage and base liberation (Rhaese and Freese, 1968; Brawn and Fridovich, 1981; Muehelmater *et al*, 1988; Schneider *et al*, 1988). This is exactly the type of damage which Bloom's syndrome cells are unable to repair due to their ligase deficiency. The high frequency of chromosome abnormalities in BS cells may be induced by similar pathways to those

stimulated by tumour promoters. Inhibitors of tumour promotion, such as protease inhibitors, inhibit the promoter induced formation of sister chromatid exchanges (Kinsella and Radman, 1978; Marx, 1983). These same protease inhibiters can also reduce the frequency of spontaneously occurring chromosome abnormalities in BS cells (Kennedy *et al*, 1983). Thus, the high frequency of chromosome abberations in BS cells may arise as a result of their inability to repair DNA nicks and breaks caused by oxygen radicals generated during natural cellular processes (The danger from these radicals in BS cells, may be increased due to the cell's reported abnormal oxygen metabolism (Cerruti, 1982)). The mechanism by which these unrepaired lesions lead to SCE's is unclear, however. BS cells show an increased (up to 100-fold) level of a set of proteins usually synthesised by cells in response to treatment with phorbol esters (Mallick *et al*, 1982). It may be that one or more of these proteins is a DNA repair enzyme, capable of catalysing genetic recombinations, which is synthesised in response to an abnormally high level of DNA strand breakage.

The existence of inducible DNA repair enzymes, with recombinogenic activity, is well known in prokaryotes (Wintersberger, 1982; Ganesan *et al*, 1982; Little, 1983). These have complex induction mechanisms with a graded response towards different levels of damage (Smith, 1985). In the yeast *Saccharomyces cerevisiae* the *RAD52* gene, required for mating type switching (an interchromosomal recombination event) is also required for recombination repair (Wintersberger, 1982) and the *RAD1* gene, one of the genes involved in excision repair, is also involved in intrachromosomal recombination.

Although they have not yet been shown, it would be surprising

if similar recombinogenic enzymes did not exist in mammalian cells (Shiraishi, 1985; Kinsella and Radman, 1972), and the involvement of inducible repair systems in carcinogenesis has been hypothesised (D'Ambrosio and Setlow, 1976; Wintersberger, 1982; Cairns, 1981; Echols, 1981).

Given that large scale changes in chromosome structure, involving translocations, transpositions and deletions, appear to be of vital importance in the development of human cancers, a better understanding of the mechanisms and factors affecting the frequency of genetic transpositions and recombinations in both prokaryotes and eukaryotes appears necessary if we are to gain a higher understanding of the events leading to the development of cancer in man.

1.2. The Transposition Phenomenon

1.2.1. Transposons and Transposition.

The existance of transposable genetic elements was first proposed by Barbera McClintock to explain her observations of unstable mutable loci in chromosome 9 of *Zea mays*. The appearence of (e.g.) pale yellow spots on a white seedling was suggested to be associated with controlling elements distinct from the loci which they affected and having no defined position relative to other genetic markers (thus implying a mechanism by which they could move within the genome).

The later discovery of certain mutants of *Escherichaie coli*, whose reversion frequency was not raised by any conventional mutagens, led to the discovery of bacterial insertion sequences and transposons (Saedler and Starlinger, 1967). These fall into three main groups: (i) Insertion sequences and

composite transposons. (ii) The transposon 3 family of transposons and (iii) Transposing bacteriophages.

Insertion sequences are small pieces of DNA (750-1500bp in length) with short, inverted terminal repeats (15-25bp), coding for the proteins necessary for their own transposition and regulation on overlapping open reading frames on opposite strands (other, host-encoded proteins, such as DNA polymerase, are often required). Composite transposons consist of two identical, or almost identical, inverted insertion sequences flanking a central region usually several thousand bp long and carrying a marker such as an antibiotic resistance gene.

The two best characterised composite transposons, Tn5 (figure 1.6) and Tn10 have non-identical insertion sequence ends due to the promoter for the central region marker gene (neomycin phosphotransferase and tetracyclin resistance respectively) having been integrated into the left hand element (IS50L and IS10L respectively). IS50R And IS10R encode the transposition functions.

The control of Tn5 transposition is quite complex. IS50R encodes two proteins, p1 and p2, from the same reading frame. p1 is a transposase protein and p2 is a repressor which suppresses Tn5 transposition by an unknown mechanism (Johnson *et al*, 1982). There are 3 promoters for these functions, T1, T2 and T3. T1 is responsible for the promotion of p1 expression and T2 (and possibly T3) promotes p2 (Krebs and Reznikoff, 1986). The levels of T1 and T3 transcripts are 100x lower than those from T2, probably due to the substitution of a C for an A in the -10 coding region of T1 and the presence of two *dam* and one *dcm* methylation sites (*dam* methylation

lowers the activity of the Tn10 pIN (transposase) promoter and is known to regulate Tn5 transposition by controlling transcriptional initiation of p1 (Yin *et al.* 1988)).

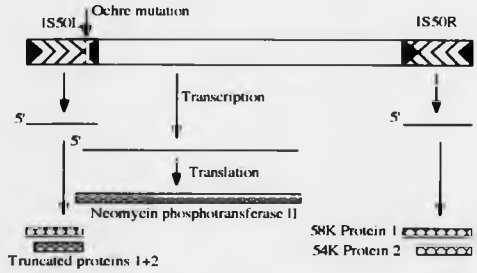


Figure 1.6. Tn5 and its gene products, the Km resistance protein neomycin phosphotransferase II, the transposase protein 1 (tnp) and the inhibitor protein 2 (inh). The truncated proteins 1 and 2 are inactive

The Tn3 family of transposons comprises about 20 elements of between 5 and 16.5Kb in length, carrying genes for antibiotic resistance. They do not have any independent IS elements at their ends. The elements encode two transposase functions on two open reading frames with two possible orientations, and a site associated with the *tnpR* function (*res*) (figure 1.7).

tnpA Encodes the transposase and *tnpR* encodes a protein with two functions, a resolvase function and a repressor function. The resolvase function catalyses the resolution of co-integrates formed by transposition between replicons. The resolvase is an endonuclease which, in the γδ element, cuts between the second and third base pairs of the sequence TTATAA.

forming a double-stranded break and allowing efficient strand exchange. Resolution requires the interaction of two *res*-resolvase complexes and thus only occurs in co-integrates (Reed and Grindley, 1981).

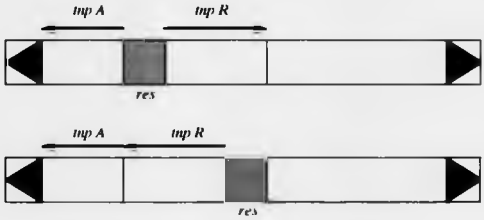


Figure 1.7. Two possible orientations of the *tnpA* (transposase) and *tnpR* (resolvase) functions in the Tn3 family of transposons. Typified by Tn3 (top) and Tn501 (bottom).

The repressor function of the *tnpR* product is mediated by its binding to sites in *res* which overlap with the promoter for *tnpA* and *tnpR* (Grindley *et al*, 1982). Transposition is further controlled by the inefficiency of translation of the *tnpA* message, probably due to the relatively high percentage of G residues in the translation initiation site, which may lower the efficiency of initiation (Gold *et al*, 1981).

The final family of bacterial transposons is the two related transposing bacteriophage Mu and D108, which share a 95% sequence homology and similar functional organisation, though infection with either of the two does not confer immunity to the second on the host.

Mu is a temperate bacteriophage similar in appearance to bacteriophage P2, with an icosahedral head 540Å wide, a contractile tail

sheath 1000Å long and 180Å wide and a base plate with tail spikes (Howe and Bade, 1975). Approximately 2% of lysogens arising following infection of *E. coli* K12 show nutritional requirements due to the insertion of the bacteriophage genome into the inactivated gene (*ibid*), leading to its name Mu (for mutator).

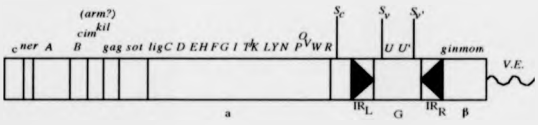


Figure 1.8. Genetic map of bacteriophage Mu. Early functions include *c* the repressor and *A* and *B* the transposase functions. IR_L and IR_R are the inverted repeats flanking the invertable G segment. *Sv* and *U*, and *Sv'* and *U'*, are the host range-determining protein products of opposite orientations of the G segment. *gin* And *mom* encode the G segment invertase and Mu modification enzymes respectively.

During lysogeny the phage is inserted into the host genome by simple insertion (ie. not involving replication of the entire Mu genome (Harshey, 1984) generating a short target sequence duplication at the site of insertion. During the lytic cycle however, Mu undergoes replicative transposition, again generating target sequence duplications, but the major product in this case being cointegrates where the the transposon-donor molecule and target molecule are joined with duplicate copies of the transposon at the junctions (Craigie and Mizuuchi, 1985).

Two Mu gene products are required for transposition. The *A* gene product is the transposase function, absolutely required for transposition. It is an endonuclease which introduces single stranded cuts at

the Mu ends, leading to 3'-OH termini. In the subsequent strand transfer step the MuA-Mu DNA complex cuts the target DNA and joins the the 3'-OH to the target 5' phosphate in a 1 step transesterification reaction with the 3'-OH as the attacking nucleophile (Mizuuchi and Adzuma, 1991).

The Mu *B* gene product enhances the efficiency of replicative transposition by 100-fold during the lytic cycle. The protein is an ATPase which preferentially stimulates intermolecular DNA strand transfer, the protein, ATP and target DNA are captured by the donor DNA-protein complex. ATP hydrolysis is enhanced after an ATP stimulated oligomerisation of B protein. ATPase activity is also modulated by DNA and A protein, being repressed in the presence of DNA alone, and greatly enhanced in the presence of both DNA and A protein (Maxwell *et al*, 1987; Adzuma and Mizuuchi, 1991).

A further Mu function associated with replicative transposition is *arm* which stimulates transposition by increasing the levels of A and B proteins (Goosen *et al*, 1982).

Also required for transposition are the two Mu ends which, unlike those of insertion sequences, are not equivalent and must be present in the correct orientation (Howe and Schumm, 1980). A sequence almost identical to the first 31 bases of the c end does appear, in three separate blocks, in the S end. Nucleotides 1-14 of c appear at 79-92 in S while nucleotides 15-31 of c appear at positions 1-20 of S in two blocks separated by a GC rich loop (Kahmann and Kamp, 1979). These sequences are the Mu *attL* and *attR* sites. *attL* Was found to contain two transposase binding regions essential for transposition (L1 and L3) and *attR* one essential region (R2), each containing the consensus sequence YGtTTCAYtNNAARYRCGAAAA (Y=purine, R=pyrimidine), a further

consensus sequence is found as an inverted repeat in *attL*. Two further weak transposase binding sites, L2 and R1 are the more mechanistically important binding sites, mutations in these two having greater effects on transposition than in L1, L3 or R2, binding of transposase to these three sites is therefore proposed to be cooperative to the binding at the weaker sites (Groenen and van de Putte, 1986).

Host functions required for Mu transposition include the replication initiation function *dnaC*, DNA polymerase III (DNA polymerase I does not appear to be required as transposition frequencies are unaltered in *polA*- strains (Oskolkova *et al*, 1984)), and the products of the *dnaB*, *dnaZ* and *dnaG* genes. Also required for the lytic phase of development (though not for Mu integration (Craigie *et al*, 1985)) is the integration host factor (IHF) (*himA*, *himI)* products), required for integration of λ_{att} which positively regulates the early Mu promoter.

The host range of Mu is determined by the orientation of the invertible G segment. This segment is 3kb long and flanked by 34bp inverted repeats. In one (G+) orientation the segment confers infectivity towards *E. coli* K12 and B, *Salmonella typhimurium* and *Arizona* while *E. coli* C, *Citrobacter freundii* and *Shigella sonnei* are G- sensitive (Toussaint and Resibois, 1983). The C terminus of the tail fibre component S is encoded by the by the left end of G and thus differs between G orientations (S_y and S'_y corresponding to G+ and G- respectively). In addition G encodes two proteins, U and U', which are expressed in the G+ and G- orientations respectively. U and U' are also incorporated into the tail fibres where they are involved in recognition and binding to cell surface oligosaccharide antigens

(Kamp *et al*, 1984). Inversion of the G segment is controlled by the *gin* product. The G segment/*gin* system of infectivity variation is analogous to the C segment/*cin* system in the P1 phage, comparisons to the P/*pin* in *E. coli* and *hin* in *S. typhimurium* have been discussed (Iida *et al*, 1984; Plasterk and Van de Putte, 1984).

Transposition of Mu may result in a number of host chromosomal rearrangements including deletions, inversions duplications and transposition of host genes. Both deletion and inversion can occur in *cis* or *trans* (ie. at the site of prophage insertion or at a site distant from the insertion). *Cis* occurring events are Mu B protein independent while *trans* events have an absolute requirement for B (Faelen *et al*, 1978).

Mu can also undergo two processes which do not rearrange host DNA as such, simple insertion and excision. Rare simple insertions may occur by an imbalance of transposition functions (Harshey, 1983). Mu excision, which may be precise (absolutely requiring Mu *A* product) or imprecise (stimulated by *A* product), is the result of homologous recombination (host *recA* dependent) (Desmet *et al*, 1980; Khatoon and Bukhari, 1981).

Several models of transposition have been proposed to explain the event and its consequences (Bukhari, 1981).

Work involving the *in vitro* transposition of a mini-Mu plasmid into the replicative form of the phage ϕX174 showed, by blocking DNA replication and freezing the transposition intermediate, that the structure of the intermediate was exactly as predicted by a model proposed by Shapiro (1979) (figure 1.9), thus greatly favouring this particular model (Craigie and Mizuuchi, 1985).

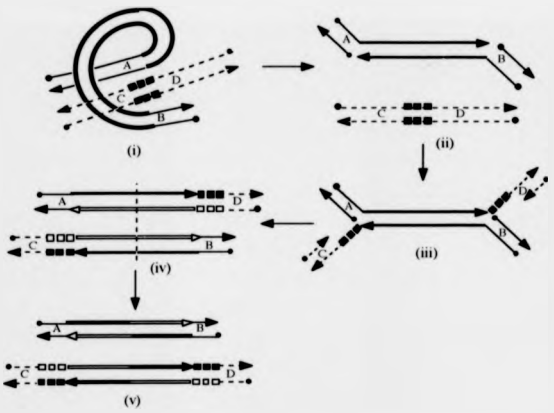


Figure 1.9. The Shapiro model for replicative recombination. (i) Association of target and donor DNA, the orientation of association determines the end products, a clockwise orientation of markers around the replicon (ABCD) results in a deletion, while an opposite orientation of C and D results in inversion on transposition. (ii) A four strand nicking of DNA occurs at opposite ends of the transposon and target sequence. (iii) Ligation of the 5' transposon ends to the 3' target strand ends occurs, forming a replication fork-like structure. The resulting products of semi-conservative replication either remain in the new orientation (A-D and C-B) (iv), or undergo a resolving recombination to regenerate the two original molecules, each with a copy of the transposon (v).

1.2.2 Mobile Genetic Elements in Eukaryotes.

The controlling elements of *Zea mays* were the first transposable elements identified (McClintock, 1950; McClintock, 1956), yielding unstable mutants of loci adjacent to their point of insertion. There are two classes of element, autonomous and non-autonomous, the first capable of acting independently and the second requiring the presence of the first. Three families of non-autonomous elements and their associated activating elements have been described (table 1.4)

Table 1.4. The three families of *Zea mays* controlling elements.

Family	Autonomous	Non-autonomous	Reference
Ac-Ds	Activator *(Ac)* Modulator *(Mp)*	Dissociation *(Ds)*	1 2
Spm	Suppressor-mutator *(Spm)* Enhancer *(En)*	No name Inhibitor *(I)*	1 3
Dt	Dotted *(Dt)*	No name	1

1. McClintock, 1950; McClintock, 1956.

2. Barclay and Brink, 1954.

3. Peterson, 1965.

The *Ac-Ds* system and the independently discovered *Mp* element (which is very similar to *Ac*) are the most studied family. *Ds* acts as a

site for specific chromosomal breakage in the presence of *Ac* (McClintock, 1946) and promotes the formation of acentric and dicentric chromosomes, via the interaction of sister chromatids at the site of *Ds* insertion, in a breakage-fusion cycle (Federoff, 1983). *Ds* elements also transpose in a replicative fashion, although not independently of chromosome replication. This may result in chromosome duplications (either direct or inverted) whose endpoints coincide directly with the *Ds* donor and recipient sites, indicating the retention of close contact of the *Ac-Ds* element with both sites on transposition.

Ac alone produces unstable mutations in various loci, although its major effects are associated with *Ds*. The effects of *Ac* (and *Mp*) are dose related. The presence of two or more elements causes a developmental delay in *Ac*-activated somatic mutation and the formation of abnormal chromosomes by *Ds*. Studies of *Mp* insertions at the P-RR locus, which produce medium variegated cobs in P-RR-*Mp*/*p* heterozygotes, have shown that, when two *Mp* are present, light variegated cobs are formed due to *Ac*-like dose dependency causing developmental delay in reversion. Transposition results in red sectors due to the removal of *Mp*, the majority of red and light-variegated sectors are twinned to each other and the former carry either no *Mp* or *Mp* inserted away from P-RR. This has led to the proposition of a mechanism for *Mp* transposition (Greenblat, 1974) (figure 1.10).

More recent genetic analysis has revealed the complete sequence of two *Ac* elements (*Ac7* and *Ac9*) from different loci which has shown them to be homologous, encoding three potential proteins in three overlapping reading frames (ORF's) (although ORF3, 453bp, was considered to be too short to encode a protein) (Müller-Neumann *et al*, 1985). Comparisons with *Ds* sequences have shown that they are related, *Ds* having

internal deletions but homologous ends, including inverted repeats (Donner *et al*, 1986). *Dl*, a *Ds* derived from the *b2-m2* allele, has a deletion of ORF2 with an intact ORF1, suggesting that both ORF's are required for transposition. The two ORF's do not complement in *trans* and it is therefore proposed that the two form a fusion protein via RNA splicing (*ibid*). ORF2 is also required for the *Ac* gene dosage effect (*ibid*).

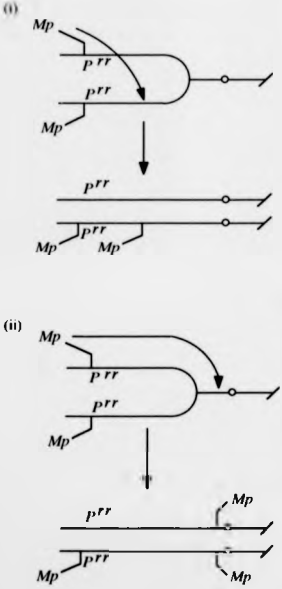


Figure 1.10. Mechanism of *Mp* transposition proposed to explain the appearance of twinned sectors. (i) Transposition of *Mp* into a previously replicated region of the sister chromatid results in a red sector of kernels carrying no *Mp* twinned with a light variegated sector carrying two *Mp*. (ii) Transposition of *Mp*, post-replication, into an unreplicated region results in red sector of kernels with *Mp* inserted away from *P-RR* twinned with a light variegated sector.

The yeast *Saccharomyces cerevisiae* possesses a group of related transposable elements at c. 30-35 copies per cell. These Ty (transposon yeast) elements consist of a central DNA (ε) region of c. 5.6Kb flanked by 330bp direct repeats (δ). A haploid yeast genome also contains over 100 copies of the δ sequences not associated with a Ty element (Cameron *et al*, 1979). There are considerable sequence inhomogeneities between Ty ε and δ regions and even greater inhomogeneities between free δ sequences (Roeder and Fink, 1983; Tschumper and Carbon, 1982).

Ty elements promote similar DNA rearrangements to those observed with prokaryotic transposons, however, these are generally the result of homologous recombination rather than transposition. Ty elements also undergo excision by recombination of the flanking δ sequences to give a circular molecule consisting of 1 δ sequence and the ε DNA, leaving the second δ at the original site (Chaleff and Fink, 1980).

Ty elements show several homologies to retroviruses, the major Ty mRNA has a terminal repeat similar to the R region of retroviral provirus transcripts and the 5' δ–ε boundary of many of the elements is complementary to the 3' end of the yeast initiator tRNA, analogous to the negative strand primer binding site of retroviruses (Elder *et al*, 1983; Varmus, 1983).

Loss of an intron during transposition of an engineered Ty element showed that Ty elements transpose via an RNA intermediate (Boeke *et al*, 1983). The Ty element Ty912 contains 2 potential protein coding sequences on overlapping reading frames. The first, *tya912*, shows sequence homologies to DNA binding proteins (Clare and Farabaugh, 1985), and the second, *tyb912* encodes the reverse transcriptase protein required for Ty

retroposition.

The fruit fly *Drosophila* possesses 3 major classes of transposable element, *foldback* (*FB*) elements, *Copia* and *copia*-like elements and *P* elements.

FB elements all have homologous inverted terminal repeats (IR's) which are extremely variable in length. The central, non-IR sequences are also variable in length and may be completely absent (Truett *et al*, 1981). Insertion of *FB* elements leads to unstable alleles of the *white* locus (Karess and Rubin, 1982; Levis *et al*, 1982).

The *copia* family of elements is the most numerous class of dispersed, repetitive elements in *Drosophila*. The elements show structural homology to retroviral proviruses (Will *et al*, 1981). Although the complete sequence of a representative *copia* element shows only weak homology to several retroviral proteins, including the reverse transcriptase, there is close homology in the region of the *pol* gene encoding the integrase function. *Copia* are more closely related to the yeast Ty elements than to retroviruses or even the *copia*-like element *17.6* which encodes an *env* product, whereas *copia* and Ty do not (Wiener *et al*, 1986). Transposition of *copia*-like elements creates a 3-5bp duplication at the target site. Although a lack of sequence homology indicates that there is no recognition sequence, some homology between *copia* and its insertion site has been reported (Dunsmuir *et al*, 1980). Element *297* does show a preference, though not a requirement, for insertion at the sequence ATAT (Rubin, 1983).

Transposition of *copia* results in insertion mutants *e.g.* of the *white* (*white apricot*) and *bithorax* (*bx*) loci (Levis *et al*, 1982; Rubin, 1983). These mutants are unstable and may revert by imprecise excision (*ibid*).

P elements are associated with the hybrid dysgenesis system of *Drosophila*. They vary in size, the smallest about 0.5kb, and all appear to be descended, via internal deletions, from a 2.9kb ancestral *P* element which encodes a *trans*-acting factor catalysing its own transposition and that of the smaller elements (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Evidence for *P* transposition comes from the mutational instability of dysgenic strains (Simmons and Limm, 1980). *P* elements inserted in the *white* locus cluster at a specific site, suggesting a recognition sequence. The *P* element transposase is an 87kD protein encoded by four ORF's, the major transcript in somatic cells is an inactive 66kD polypeptide derived from the first three ORF's, germline specificity of the dysgenic effect is thus thought to be due to efficient removal of the final transposase intron in germ cell mRNA (Laski *et al*, 1986). The transposase catalyses precise and imprecise, non-autonomous *P* element excision and reintegration.

Mammalian genomes contain large numbers of non-retroviral transposing elements. These can broadly be separated into four categories, viral-like retroposons, retropseudogenes, long interspersed repeated sequences (LINES) and short interspersed repeated sequences (SINES).

A viral-like, putative transposon THE-1 (transposable human element) has been described in human cell lines. This element shows many features of a transposable element. THE-1 is 2.3kb in length, has two flanking 350bp, LTR-like sequences, it is flanked by 5bp direct repeats and is present at about 10 thousand copies in the human chromosome. THE-1 is also present in extrachromosomal closed circular DNA's and mRNA transcripts of discrete length, homologous to THE-1 are present in HeLa cells (Paulson *et al*, 1985). THE-1 shows no homology to any known retrovirus (*ibid*).

Retropseudogenes are derived from processed mRNA transcripts. All classes of cellular RNA except the four ribosomal rRNA species give rise to retropseudogenes. These retropseudogenes resemble cDNA copies of fully processed mRNA parents. They often extend to the 5' cap site (although 5' deletions are not uncommon), they have no introns and always include the 3' poly(A) tract of the parental species (Weiner *et al*, 1986). Retroposition of correctly initiated mRNA nearly always leads to an inactive gene due to the loss of the untranscribed promoter sequences, the human metallothionein II retropseudogene is inactive despite an intact coding region (Karin and Richards, 1982; Karin *et al*, 1984). Such inactive retropseudogenes are subject to degeneration due to neutral drift. Occasional insertion adjacent to a foreign promoter may result in an active gene. This may be the origin of the intronless chicken calmodulin gene (Weiner *et al*, 1985). Additionally, the rare retroposition of an aberrant mRNA, including promoter sequences, may lead to a functional gene, the rat and mouse preproinsulin I gene is derived from a partly processed, aberrant mRNA and contains only one of the parental gene's two introns (Soares *et al*, 1985).

The mammalian genome contains 20-50 thousand copies of the LINE1 (L1) family of elements. Each species has a related but species-specific family, each of which may be considered a superabundant and complex family of retropseudogenes. Some L1 elements may be functional retropseudogenes (Weiner *et al*, 1985). Most L1 elements end with an A-rich tract and contain one or more ORF's. They are mobile and make target site duplications on insertion. A variety of polymorphisms associated with L1 insertion or deletion suggest that contemporary L1 elements may retropose, moreover, 6.5kb poly adenylated transcripts homologous to L1 are found in the cytoplasm of the

relatively undifferentiated human NTera2 cell line and a reverse transcriptase activity, associated with a viral-like complex with a 37kD protein major component, has been detected in these cells. This activity may be L1 transcript-encoded, or may be involved in its retroposition (Deragon *et al*, 1990).

SINE elements are typified by the human *Alu* family, of which there are 500 thousand copies in the human genome (comprising a remarkable 5-6% of the genomic mass). These are 300bp long dimeric retropseudogenes derived from 7SL RNA by one or more internal deletions followed by dimerisation. The right monomer is 31 nucleotides longer than the left due to more extensive internal deletion of the left monomer. The elements only transpose as a dimer, probably due to an inactivated promoter in the right monomer (the internal promoter defines the 5' end of the *Alu* element by directing RNA polymerase III transcription to a defined upstream position) although this is slightly counter-intuitive as the right monomer more closely resembles the parental 7SL RNA (Weiner *et al*, 1985). The super abundance of *Alu* and other SINE families is attributed to the ability of many or all newly retroposed elements to act as templates for further retroposition. This would be expected however to result in a shortening of the 3' A-rich tract, presumably the primer site for reverse transcription, at each round. This has not occurred but it is possible that the A-rich region is expanded, post-retroposition, by a mechanism analogous to that which is thought to cause the expansion of simple satellite sequences (Rogers, 1983), similar expansions of the A-rich region of the left *Alu* monomer have been reported (Weiner *et al*, 1985).

The mechanism of non-viral retroposition remains elusive and, apart from the observation that it involves the insertion of RNA information

into a staggered chromosomal break, there is little consensus of opinion as to the mechanism at work (there may be more than one although it is thought possible to reconcile all known forms of non-viral retroposition with a single mechanism (*ibid*)).

Retropseudogenes are relatively rare in vertebrates other than mammals, this as been attributed to the prolonged periods spent by mammalian oocytes in the lampbrush stage of development (up to 40 years in humans compared to < 3 weeks in birds) (*ibid*). If this is the case and retroposition occurs predominantly in the female germline, then retropseudogenes may be expected to be less common in the male-specific Y chromosome.

The existence of such large numbers of mobile genetic elements in mammalian cells raises the question of their evolutionary significance. The possession of a relatively fluid genome, with the generation of novel genes, pseudogenes and transposons, may confer a selective advantage to the organism, however, the possible negative aspects of unfavourable genetic damage and possible carcinogenesis, due to insufficient negative regulation, cannot be ignored, insertional mutations of the *myc* locus by L1 elements have been reported in human breast carcinomas (Morse *et al*, 1988).

1.3. The Development of a Short-Term Test for Carcinogens Based on Bacterial Transposition.

1.3.1. The Nature of the Test.

Following initial, unpublished observations by B.E.P. Swoboda and B.S. Hayer in 1983, that the potent carcinogen N-methyl-N-nitro-N-nitrosoguanidine (MNNG) was able to enhance the frequency of transposition

of the bacteriophage Mud1(Ap^r, *lac*), two systems were constructed with the aim of investigating the effects of known and potential carcinogens on the *in vivo* transposition frequencies of Mud1 and Tn5 (Wilkins, 1987) (see also section 1.2.1), with a view towards a potential new test for carcinogenic chemicals.

The first system involved the Mud1(Ap^r, *lac*) construct of bacteriophage Mu (Casadaban and Cohen, 1979) (Figure 1.11).

Figure 1.11. Genetic map of Mud1(Ap^r, *lac*). Ap^r: gene for ampicillin resistance; *lac A*, *Y* and *Z*; genes of the lactose operon derived from Tn3-81.

The Mud1(Ap^r, *lac*) construct is defective due to the deletion of several essential genes and their replacement with a Tn3 derivative (Tn3-81), carrying the Tn3 ampicillin resistance gene *bla* (Ap^r) and an internal *ara*-Mu'-*trp-lac*$^+$ insert. The construct was introduced into an *E. coli* strain, MC4100, with a *lac* operon deletion (*i.e.* phenotypically *lac*$^-$), at a site where the construct's internal *lac* operon (which lacks it's own promoter/operator sequences) is not expressed by any external promoters.

Subsequent transposition of the transposition-competent Mud1 construct carries a small probability of insertion next to a promoter capable of directing transcription of the internal *lac* operon, and expression of a *lac*+ phenotype. Reversion of MC4100 Mud1(Ap^r, *lac*) to a lactose fermenting

phenotype can thus be used to give an estimate of the transposition frequency (tf) of the Mud1 construct (N.B. the *actual* tf will be 1-2 orders of magnitude higher than the frequency of *lac*$^+$ reversion, as only a small percentage of transpositions result in *lac*$^+$ expression).

The second system constructed involved a strain of *E. coli*, UWE103, with Tn5 inserted into its genome. The strain was transformed with the multicopy plasmid pBR322 to provide multiple targets for Tn5 transposition. Transposition of Tn5 results in an increase in the copy number of the Tn5 encoded neomycin phosphotransferase II gene which confers resistance to the antibiotic kanamycin (km). The increased copy number of the gene theoretically allows the organism to survive in the presence of much higher levels of km than strains carrying only a single copy. Tf can therefore be monitored by measuring the number of organisms resistant to 1000μg ml^{-1} km, compared to those resistant to km at 50μg ml^{-1}.

1.3.2. Initial Results Obtained.

Over a three year period, the bacterial tester strains and the methodology for their utilisation were developed (Wilkins, 1987; Wilkins and Swoboda, 1987a) and a number of chemicals tested for their ability to affect the transposition frequency of the relevant transposon (a large majority of the work was performed using the first tester strain developed, MC4100 Mud1(Ap^r, *lac*$^+$)). A number of interesting results were obtained.

Following the initial observations of Swoboda and Hayer, the alkylating nitro-nitrosoguanidines (NNG's) (methyl, ethyl and propyl-nitro-nitrosoguanidines) were found to be highly transposogenic, inducing 3-5 fold increases in tf of Mud1(Ap^r, *lac*$^+$) at doses of $\leq$ 60μg of chemical per test plate.

MNNG, also caused similar levels of Tn5 tf enhancement. The alkyl-NNG's were the most potent enhancers of tf discovered. Of the other alkylating agents tested, four gave positive increases in tf with Mud1(Ap^r, *lac*), these were Ethyl methanesulphonate (EMS), methyl nitrosourea (MNU), Ethyl nitrosourea (ENU) and dimethyl nitrosamine (DMN). Three further alkylating agents, methyl methanesulphonate (MMS), diethyl nitrosamine (DEN) and dipropyl nitrosamine (DPN) (all mutagens in the Ames *Salmonella* mutagenicity test) gave very low enhancements or no enhancement of the tf of Mud1(Ap^r, *lac*).

The different levels of enhancement of tf by the alkylating agents was initially attributed to the differing types of alkylation damage caused by each. Transposogenicity was linked to the ability to cause O^6-alkylation of guanine, lack of transposogenicity was due to an inability to undergo the S_N2 type mechanism required for O^6-alkylation of guanine (MMS) or due to a requirement for metabolic activation for mutagenicity (DEN, DPN). Following further investigation however, a second hypothesis was suggested to explain the induction of transposition by these chemicals. It was proposed that the ultimate inducer of transposition in each case, by an unknown mechanism, was guanosine-3',5'-cyclic monophosphate (cGMP) (Wilkins, 1987; Wilkins and Swoboda, 1987).

Several observations supported this hypothesis. Firstly, MNNG, which is known to raise intracellular levels of cGMP in rats by a nitroxide radical (NO) mediated activation of guanylate cyclase, also raised cGMP concentrations in the tester strain MC4100Mud1(Ap^r, *lac*) by a comparable degree. Secondly, an enhancement of the MNNG effect was observed in the

presence of the cyclic guanosine phosphodiesterase inhibitor M&B 22948, and a corresponding antagonistic effect by methylene blue, which blocks the NO -dependent induction of rat guanylate cyclase by MNNG was reported. Thirdly, another NO generating chemical, amyl nitrite, produced a small enhancement of Mud1(Ap^r, *lac*) tf. Finally an elevation of tf in both tester strains, by membrane permeable derivatives of cGMP was reported, once again M&B 22948 agonised the effect of cGMP on Mud1(Ap^r, *lac*).

In addition to the above observations, the effect on the transposition of both Mud1(Ap^r, *lac*) and Tn5 by a variety of metal ions was investigated. In both cases Cr(IV), Mn(II) and Cd(II) were transposogenic and Co(II) appeared to have a negative effect on transposition (*i.e.* tfs were lower in the presence of Co(II)). Cb(III) was non-transposogenic to Mud1(Ap^r, *lac*) and Ni(II) was transposogenic to Mud1(Ap^r, *lac*), though a high cell kill at the transposogenic doses brought the positive classification of the metal into some doubt. The trasposogenicity of the metal ions tested appeared to correlate well with their microbial mutagenicity.

The effects of five other chemicals on the transposition of Mud1(Ap^r, *lac*) were also reported. The DNA gyrase inhibitor oxolinic acid caused a significant decrease in tf, attributed to the reduction in the levels of the tight, positive DNA supercoils required as a target for Mu transposition. Xanthine (X), in the presence of xanthine oxidase, produced a small increase in Mud1(Ap^r, *lac*) tf. This effect was diminished when superoxide dismutase was also added and was thus due to superoxide radicals (O_2^-) produced in the reduction of xanthine to uric acid by xanthine oxidase. As previously discussed (1.1.3) oxygen radicals cause DNA strand scission, backbone

breakage and base liberation (Rhaese and Freese, 1968; Brown and Fridovich, 1981; Muehelmater *et al*, 1988; Schneider *et al*, 1988). Such damage would appear to be transposogenic. However, the observed elevation of tf was low (possibly due to the fact that *E. coli* cell membrane is impermeable to oxygen free radicals) and the observation clearly warrants further investigation.

Finally, three non-mutagenic carcinogens, diethyl stilbesterol (DES), dichloro 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane (DDT) and ortho-toluidine, gave some enhancement in Mud1(Apr, *lac*) tf. No convincing explanation for the transposogenicity of these non-mutagenic chemicals was found, however, the ability of the transposition tester strain to identify them as having a measurable effect on DNA structure raised the exciting possibility of a short-term test for both mutagenic and non-mutagenic carcinogens.

1.4. Aims of Project and Initial Direction of Research.

1.4.1. Aims.

The research reported hereafter was undertaken with both short and longer-term aims in mind. The short-term aims were as follows:

(i) Further investigation into the reported effect of cGMP on the transposition frequencies of both Mud1(Apr, *lac*) and Tn5, with an eye towards gaining some insight into the mechanism of cGMP action.

(ii) Expanding the range of chemicals tested, initially to those capable of raising intracellular levels of superoxide radicals in order to see if the clastogenic effects of these radicals were reflected in an increase of tf. Radical forming species are often difficult to detect as mutagenic in conventional short-term tests.

(iii) Development of the transposition tests, by improvements in both the methodology and the bacterial tester strains used, in order to increase the sensitivity of the test towards any transposogenic effects of the chemicals tested. As the test stood, sensitivity was very low, with only one group of chemicals, reported as being transposogenic (the NNG's), producing greater than a doubling in tf. For any potential test for carcinogens, a *minimum* of doubling in the observed effect (*his* reversion, interchromosomal recombination, sister chromatid exchange *etc*.) is the widely accepted requirement for the classification of any test chemical as 'positive' in that test.

The above short-term aims were designed with one longer-term aim in mind, the development of a novel, short-term test, based on the induction of large scale changes in DNA structure, capable of identifying both mutagenic and non-mutagenic (*i.e.* not detected as mutagenic in conventional short-term tests but shown to induce cancers in animal tests) carcinogens. Ideally the test would also reflect a difference in the mechanism of action of the two classes of compound.

1.4.2. Initial Direction of Research.

It was decided that the initial work would involve repetition of the experiments which had most strongly suggested the involvement of cGMP in the induction of transposition. Wilkins (1987) had reported the results shown in table 1.5 using the membrane permeable cGMP derivative $N^2,O^{2'}$-dibutyryl guanosine-3',5'-cyclic monophosphoric acid (dbcGMP).

Two things should be noted from these results. Firstly, the effect of dbcGMP on both systems was almost identical, secondly, the level of deviation in the results (three duplicates at each dose) was very low, $\leq 1.5\%$ in

each case. A consistently low SD in Wilkins' experiments enabled very small effects on tf to be classed as statistically significant when the student's T-test was applied to the data. This low level of standard deviation however, was seldom achieved in the experiments reported herein or by other workers using the two systems.

Table 1.5. Effect of mbcGMP on tf of Mud1(Apr, *lac*) and Tn5 (Wilkins, 1987).

cGMP (μg plate^{-1})	Relative Tf (Mud1(Apr, *lac*))[a]	Relative Tf (Tn5)[a]
0	1.00±0.01	1.00±0.01
12.5	1.28±0.01	1.28±0.01
25	1.47±0.01	1.58±0.01
50	1.99±0.01	2.07±0.01

a. Tf relative to control experiment ± standard deviation (3 duplicates).

It was decided to try to confirm these results by replication and follow up the observations using db derivatives of other cyclic nuceotides (Wilkins also reported a very small depression in the tf of Mud1(Apr, *lac*) in the presence of dbcAMP) and other potential agonists and antagonists of cGMP on both transposition systems.

Other experiments to be undertaken were the testing of the effect of oxygen free radicals on transposition of both systems. The chemical chosen to study first was methyl viologen (paraquat). In *E. coli*, PQ undergoes a redox cycle whereby it is reduced by an NADPH dependent diaphorase enzyme and subsequently reoxidised *in situ* from molecular

oxygen. PQ is therefore a convenient tool for specifically raising intracellular levels of O_2^- (for a more detailed description of the *in vivo* effects of PQ see section 6.2.3).

CHAPTER 2: MATERIALS AND METHODS.

2.1. Materials.

2.1.1. General Usage Chemicals.

The following chemicals were obtained from the Sigma chemical company: α–lactose hydrate (substantially glucose free); α-D(+)glucose (anhydrous, grade 3); D(+)galactose (<0.01% glucose); L(+)arabinose, ampicillin (sodium salt); tetracycline hydrochloride; gentamycin sulphate; Trizma (tris) base; d-biotin; uracil; adenine hydrochloride; L-leucine; L-isoleucine; L-valine; L-methionine; L-proline; glass beads (425-600μm); ethyleneglycol-bis-(β-amino-ethylether) N,N'-tetra acetic acid (EGTA); xanthine (sodium salt); nitrofurantoin; thiamine hydrochloride; sodium ammonium phosphate; haematein (from bovine blood); actinomycin D (from *Streptomyces* species).

Fisons scientific equipment were the suppliers of dimethylsulphoxide (DMSO); triethanolamine; potassium dihydrogen orthophosphate; dipotassium hydrogen orthophosphate; sodium azide; glycerol; D-glucose (anhydrous); magnesium sulphate; sodium chloride (all SLR grade); ferrous sulphate (AR grade).

Difco Laboratories supplied agar noble; bacto peptone; MacConkey agar base; yeast nitrogen base (w/o amino acids, dehydrated).

Nutrient broth and agar technical (No. 3) were obtained from Oxoid Ltd.

British Drug Houses (BDH) Ltd were the suppliers of L-tryptophan; L-lysine monohydrochloride; L-histidine monohydrochloride; L-arginine monohydrochloride; potassium hydroxide pellets; sodium dihydrogen

orthophosphate; di-sodium hydrogen orthophosphate; crystal violet.

Powdered yeast extract was obtained from London Analytical and Bacteriological Media Ltd (Lab M).

May and Baker Ltd supplied citric acid.

Sodium thiosulphate (photo grade) was obtained from Koch-Light Laboratories Ltd.

Finally, ethylenediaminetetra acetic acid (disodium salt)(EDTA) and ammonium sulphate were obtained from Hopkin and Williams.

2.1.2. Chemicals Tested in Transposition Assays.

From Sigma: N^2,$O^{2'}$-dibutyryl guanosine-3',5'-cyclic monophosphoric acid (dbcGMP)(sodium salt)(lot nos 47C-7240, 45F-7150, 85F-7126); N^2-monobutyryl guanosine-3',5'-cyclic monophosphoric acid (N-mbcGMP)(sodium salt)(lot no 89C-7490); N^{6}-2'-O-dibutyryl adenine-3',5'-cyclic monophosphoric acid (dbcAMP)(mono sodium salt)(lot nos 16F-7156, 104F-7238-1); O^2-monobutyryl adenine-3',5'-cyclic monophosphoric acid (mbcAMP)(sodium salt)(lot no 40F-7220); O^2-monobutyryl inosine-3',5'-cyclic monophosphoric acid (mbcIMP)(sodium salt)(lot no 33C-7250); N-methyl-N'-nitro-N-nitroso guanidine (MNNG) (lot No 112F-0003); 1,1'-dimethyl-4-4'-bipyridinium dichloride (methyl viologen, paraquat) (lot Nos 25F-3535, 16F-3400, 127F-3506); phorbol-12-myristate-13-acetate (PMA) (lot No 46F-0269); mitomycin-C (from *Streptomyces caespitosis*) (lot No 96F-0547-1).

From BDH: Sodium nitroprusside (Analar) (lot No 444195/5705).

From Fisons: Dimethylsulphoxide (batch 75), triethanolamine (batch A50).

From The Protein Research Foundation: Antipain (lot No 110709).

The cyclic guanosine monophosphate phosphodiesterase inhibitor, M&B 22948, was obtained as a gift from May and Baker. Sodium acetate (anhydrous) was also obtained from M&B.

2.1.3. Recipes for Growth Media.

M63 salts (5 x concentrated M63 medium).

68.0g potassium dihydrogen orthophosphate, 10.0g ammonium sulphate, 2.5mg ferrous sulphate were added respectively to 800ml distilled water and the pH was adjusted to 7.0 with potassium hydroxide. The volume was adjusted to 1 litre and autoclaved (15 psi, 15mins, 121°C).

Before use as liquid media, M63 salts were diluted 5 fold, magnesium sulphate (1M, autoclaved separately) added at 1ml per litre, and glucose (20%, autoclaved separately) added at 10ml per litre.

M63/ lactose or glucose/ ampicillin plates.

15g agar (agar noble for transposition assays, agar No.3 for general use) was added to 800ml distilled water and autoclaved (15 psi, 121°C, 15mins). After cooling to around 50°C, 1ml 1M $MgSO_4$, 10ml either 20% lactose or 20% glucose (autoclaved separately) and 1ml 25mg/ml ampicillin (filter sterilised) were added along with 200ml of warmed M63 salts. After

mixing, the agar was poured onto petri dishes (approximately 25ml per plate), allowed to cool and dried by leaving inverted overnight at room temperature, before use.

Where required, other antibiotics or growth factors were added, immediately prior to pouring as follows: per litre; gentamycin sulphate, 1ml 20mg/ml; kanamycin, 1ml 50mg/ml; threonine, 5ml 0.71%; thiamine, 5ml 0.34%; leucine, 5ml 0.79%.

Plates containing ampicillin were used within three days of preparation.

LB Medium and plates.

15g Agar, 10g bacto tryptone, 5g yeast extract, 5g sodium chloride and 2ml 1N sodium hydroxide were added to 1 litre distilled water and autoclaved (15 psi, 121°C, 15mins).After cooling to around 50°C the mixture was poured onto petri dishes as with M63/ lactose/ ampicillin plates.

Where required, the following selection agents were added immediately prior to pouring: per litre; kanamycin, either 0.5ml or 10ml (for estimation of Tn5 transposition frequency) 100mg/ml; gentamycin, 1ml 20mg/ml; nalidixic acid, 1ml 20mg/ml; tetracycline, 1ml 20mg/ml.

LB medium was prepared in the same way, with the exclusion of agar.

YPAD Medium and plates.

20g agar, 20g peptone, 10g yeast extract, and 20g glucose were made up to 1 litre with distilled water and autoclaved (15 psi, 121°C, 15mins). After cooling, 1ml 30mg/ml adenine sulphate (filter sterilised, 30mg/ml) was added and the mixture poured onto petri dishes.

YPAD medium was prepared in the same way with the exclusion of agar.

MacConkey Lactose plates.

40g MacConkey agar base and 10g lactose were made up to 1 litre with distilled water and autoclaved (15 psi, 121°C, 15mins). The mixture was cooled and poured after the addition of any selective agents.

H- broth.

8.0g nutrient broth, 5.0g peptone, 5.0g sodium chloride, 1.0g glucose and 0.25ml 1N sodium hydroxide were made up to 1 litre with distilled water and autoclaved (15 psi, 121°C, 15mins).

SC Concentrated yeast growth supplement.

0.4g of L-histidine, L-arginine, L-isoleucine, L-lysine, uracil; 0.6g L-leucine; 1.5g L-valine were made up to 160ml with distilled water and autoclaved (15 psi, 121°C, 15 mins).

20ml of adenine (20mg/ml) and 20ml L-tryptophan (20mg/ml)(both filter sterilised) were added subsequently.

SC-leucine, SC-histidine and SC-adenine were prepared in the same way, with the omission of the corresponding supplement.

SC yeast media and plates.

6.7g yeast nitrogen base (w/o amino acids), 20g glucose and 20g agar were made up to 1 litre with distilled water and autoclaved (15psi, 121°C, 15mins). After cooling, 10ml of SC, SC-leu, SC-his, or SC-ade were added, and the mixture poured into petri dishes.

Sloppy top agar.

0.6g agar and 0.5g sodium chloride were made up to 100ml with distilled water and autoclaved (15psi, 121°C, 15mins). The solution was kept molten at 50°C until use.

For making estimates of total viable cell numbers, using M63/lactose plates, 0.5ml 20% glucose was added immediately prior to use.

Top agar for use in yeast platings was made in the same way, with the exclusion of the sodium chloride.

2.1.4. Bacterial, Bacteriophage and Yeast Strains.

Table 2.1. Bacterial strains employed.

***Escherichiae coli* strains**

Strain	Genotype	Source	Reference
K12	Wild type: No auxotropic markers	Departmental stock	
MC4100 MudI(Ap^r, *lac*) isolate a$_4$	F, *ara*D139, Δ(*lac*IPOZYA), U169, *thi*, *str*A, Mu cts dI(Ap^r, *lac*)	Departmental stock	Wilkins (1987)
MAL103	F, Mu cts dI (Ap^r, *lac*), Mu cts 62, Δ(*pro*AB, *lac*IPOZYA) X111 *str*A	Departmental Stock	Casadaban and Cohen (1979)
UWE103	*hsd* R, *ara*D139, Δ(*ara*, *leu*)7697, Δ *lea*X74(*lac* IPOZY), *gal*K, *str*A, ZZZ::Tn5 (Km^r)	Departmental stock	Wilkins (1987)

UWE103 (pBR322) isolate 3	As UWE103 carrying pBR322 (Tc^r, Ap^r)	Departmental stock	Wilkins (1987)
UWE103 (pPH1J1)	As UWE103 carrying pPH1J1 (Gm^r)	Dr. D. Hodgson, Dept. Biol. Sci., Univ. Warwick.	
UWE110	*gal*, *ara*, *lac*, *thr*, *leu*, *thi*, *ton*A, *tsx*, Tn5 (maps between *met*B and *arg*E). T6 and T1 resistant	Dr. D. Hodgson	
UWE110 (pPH1J1)	As UWE110 carrying pPH1J1 (Gm^r)	Dr. D. Hodgson	
UWE110 (pPH1J1) *rec*A R1	As UWE110 (pPH1J1), transduced to *rec*A$^-$ using T4GT7 Tn10::*rec*A		This work

MC4100 Mud1(Apr, *lac*) (pPH1J1)	As MC4100 Mud1(Apr, *lac*) carrying pPH1J1 (Gmr)		This work
GM48	As UWE110 *dcm*, *dam*		Dr. D. Hodgson
GM48 (pPH1J1)	As GM48, carrying pPH1J1 (Gmr)		This work
GM48H (pPH1J1)	GM48 (pPH1J1) selected for resistance to 1000μg ml^{-1} kanamycin		This work
FE20	F$^-$, *tru*-1, *ara*-14, *leu*B6, Δ(*gpt-pro*A)62, *lac*Y1, *tsx* 33, *sup*E44, *gal*K2, λ$^-$, rac, *his*G4, *rfb*D1, *mgl*-51, *rps*L13, *kdg*K51, *xyl*-5, *mtl*-1, *arg*E3, *thi*-1, Nal^r	Dr. D. Hodgson	
FE20 (pPH1J1)	As FE20, carrying pPH1J1 (Gmr)		This work

FE20 (pPH1J1)::Mud1 (Ap^r, *lac*)	As FE20, carrying pPH1J1::Mu d1(Ap^r, *lac*)		This work
KC89 MH3823 MH3824	F+, Mu cts 62 hP1#1, *ara*D/R751::Mu d1(Ap^r, *lac*)	KC89 from Dr. L. Csonka, Dept. Biol. Sci., Purdue Univ., Indiana 47907 USA. MH293 and 294 from Dr. M. Howe, Dept. Microbiol., Univ. Tennesse, Tennesse 38163 USA.	Csonka *et al* (1981)
JC10240	Hfr, *rec*A, *srl* C300::Tn10 (Tc^r)	Dr. G. Salmond, Dept. Biol. Sci., Univ. Warwick.	

***Salmonella typhimurium* strains**

TA1535	*his*G46, *rfa*, Δ*uvr*B	Dr. R. Tye, Severn Trent Water Authority Research Station, St Martin's Rd Coventry.	Ames *et al* (1975)
TA1950	ΔuvrB, *his*G46	Dr. B. Ames, Dept. Biochemistry, Univ. California, Berkeley, CA94720, USA.	*ibid*
TA1975	*rfa*, *his*G46	Dr. B. Ames	*ibid*
TL1S4	galE496, *met*A22, *met*E55, *rps*L120, *xyl*-440 (fels2) , H1-6 *nml* H2-*enx*, (*ilv*?), *hsd*L6, *hsd*SA29 [Mud1(Apr, *lac*), Mu cts 62hP1#1], srlC2::Tn10 *rec*A1	Dr. L. Csonka	Csonka *et al* (1981)

TA1535 Mud1(Apr, *lac*) TA1950 Mud1(Apr, *lac*) TA1975 Mud1(Apr, *lac*) isolate 10	As per parent strains. transduced with Mud1 using lysates from either KC89 or TL154		This work

The generalised transducing phage construct T4GT7 Tn10::*rec*A was obtained from Dr. G. Salmond, University of Warwick (Plakidou *et al.* 1984).

The genotype of the *Saccharomyces cerevisiae* strain RS112 (Scheistl, 1989), kindly donated by Dr. R.H. Scheistl, Dept. Biology, University of Rochester, Rochester, New York 14627, USA, was MATa/α, *ura3-52/ura3-52, leu2-3/leu2-Δ98, trp5-27/TRP5, arg4-3/ARG4, ade2-40/ade2-101, ilv1-92/ILV1, HIS3*::pRS6/*his3-Δ200, LYS2/lys2-801.*

2.1.5. Equipment.

Liquid cultures were incubated in an LH Engineering MkX incubator shaker. Centrifugation was carried out in a Sorvall RC-2B refrigerated centrifuge using SS-34 and GSA rotors, in the case of microcentrifugation, an Eppendorf 5415C variable speed microcentrifuge was employed. Sterilisation of media etc. was carried out in a Rodwell Scientific Instruments series 2001 autoclave, or a Prestige pressure cooker. Sterilin 90mm single or triple vented petri dishes were used for media plates, and incubated

in London Thermal Equipment Ltd incubators. For pipetting, Gilson P1000 and P5000 micropipettes, Socorex 5-50μl micropipettes and Oxford sampler 100μl and 50μl fixed volume micropipettes were used. Non-autoclavable solutions were filter sterilised using Flowpore D26 0.2μm sterile filter units, in conjunction with Steriseal syringes

Filter mating of bacterial cultures was carried out using a Millipore 47mm glass filter holder in conjunction with a Pyrex Buchner flask and Whatman 0.22μm nitrocellulose membrane filters. Whatman 0.6mm AA discs were used for spot testing of chemicals and antibiotics. A Techne Dri-block DB3 was used for maintaining molten top agar prior to plating of cultures. pH Was measured using an Electronic Instruments Ltd model 7030 pH meter connected to a Kent Industrial Measurements Ltd series 1160 combination pH electrode. Optical density was measured using a Pye Unicam SP1800 UV-visible spectrometer connected to a Churchill water pump and a Unicam AR25 linear recorder, or, in the case of bacterial culture density measurements, an MSE Spectroplus was used.

2.2. Methods.

2.2.1. Plate Incorporation Assay for Potentially 'Transposogenic' Chemicals, using Mud1(A_p^r, *lac*) Lysogens.

Cultures of Mud1 lysogens for transposition assays were grown by scraping off a small amount of a frozen permanent culture (made by adding 10% DMSO to an overnight culture of the lysogen in LB medium and freezing in sterile 1ml vials at -70°C) with a sterile Pasteur pipette, and using to inoculate 25ml sterile M63 medium, supplemented with 0.25ml sterile glucose

solution (20%), 25μl 25mg/ml ampicillin, 25μl 20mg/ml tetracycline, 25μl 1M $MgSO_4$ in a sterile 125ml conical flask sealed with a polyurethane bung.

The cultures were grown to mid to late log phase (OD_{600}=0.9 to 1.2) by incubation at 30°C, with shaking at 200rpm overnight. Cells were pelleted by centrifugation at 1200g, 5°C for 20 mins (10000rpm, SS34 rotor) and resuspended in 25ml fresh M63 medium.

For the estimation of cell survival, cultures of the Mud1 transposition tester strains were diluted 10^6 fold, in ten-fold steps, in sterile M63 medium. 100μl Aliquots of the 10^6 and 10^5 fold dilutions were added to 2ml sloppy top agar, kept molten in sterile test tubes at 47°C in a Dri-Block. These were immediately poured onto M63 glucose plates and spread by gentle tilting. Platings of each dilution were performed in triplicate. For the estimation of cell survival in the presence of 'transposogenic' chemicals, the above procedure was repeated with the addition of up to 100μl of the appropriate chemical solution to the top agar immediately prior to pouring.

For the estimation of transposition frequencies, the above steps were repeated using the 10^0 and 10^1 fold dilutions spread onto M63 lactose plates. In the case of chemicals with a high toxicity, or a high transposogenicity, a higher or lower dilution was used as appropriate.

After setting of the top agar (15 to 30 mins at room temperature) plates were incubated inverted at 30°C. The number of colonies on each plate was counted after 72 hours. Transposition frequencies were calculated by dividing the number of *lac*+ colonies at each chemical concentration by the total number of surviving cells at the same concentration (adjusted for the dilution factors involved).

2.2.2. Second Draft Protocol for the Plate Incorporation Assay for Potentially 'Transposogenic' Chemicals, using Mud1(A_{μ}^{r}, *lac*) Lysogens.

Initial experiments using the above protocol highlighted two minor problems with the procedure. Firstly, the amount of sloppy top agar used was often insufficient to allow even spreading across the plate, thus the volume of top agar was increased to 2.7ml. This allowed even spreading of the agar with no significant increase in setting time or effect on cell counts. Secondly, after 72 hours incubation at 30°C, the colonies on the cell survival plates grew to such a great diameter that they overlapped each other, making the counting of the number of colonies extremely difficult. This problem was easily resolved by the use of M63 lactose plates for the estimation of cell survival, glucose being added to the top agar to 0.05%. This concentration of glucose allowed the colonies to grow to a maximum diameter of 1 to 2mm, allowing an easy estimation of their numbers with no loss of viability (in fact colony counts were generally higher on these low glucose plates than for the same dilution on plates containing glucose).

2.2.3. Plate Incorporation Assay for the Estimation of Transposition Frequencies using UWE103.

Cultures of UWE103 were grown from frozen permanents, inoculated into 125ml conical flasks containing 25ml LB medium supplemented with 25μl 50μg/ml kanamycin. Cultures were grown to mid to late log phase by incubation at 37°C, 200rpm o/n. The plating procedure was identical to that in section 2.2.2. with the exception that cell survival was estimated using LB agar plates with 50μg/ml kanamycin, and transposition frequencies were estimated on LB agar/1000μg/ml plates. Plates were

incubated at 37°C and colonies counted after 72 hours. Transposition frequencies were calculated by dividing the number of colonies on the high kanamycin concentration plates by the number on the lower kanamycin concentration plates (adjusted for the dilution factors involved).

2.2.4. Subculture Protocol for the Estimation of Transposition Frequencies using All Tester Strains.

This procedure was used when the treatment of a tester strain with a potential transposogen for a defined period of time, or at a different temperature was necessary. Overnight cultures of the tester strain were grown to mid to late log phase in the appropriate culture medium, as in section 2.2.2. The culture was subsequently diluted five-fold into fresh culture medium in the required number of subculture flasks (eg. 5ml culture into 20ml fresh medium in 125ml conical flask). Any chemical to be tested was added to the subcultures at the appropriate concentrations and the flasks were incubated at the proper temperature for the required time with shaking at 200rpm.

Following the subculture step, 1.5ml of culture from each flask was removed into a sterile Eppendorf microcentrifuge tube and the cells pelleted by microcentrifugation (5min, 13000g). The cells were washed twice and resuspended in sterile M63 medium to remove any traces of chemical. Cell survival and transposition frequencies were estimated by dilution and plating as in sections 2.2.2. or 2.2.3. (without the further addition of any test chemical to the top agar).

2.2.5. Second Draft Subculture Protocol for the Estimation of Transposition Frequencies using All Tester Strains.

Use of the above protocol (2.2.4.) led to a high degree of variation in baseline transposition frequencies, even within the same experiment, leading to a poor identification of elevated post-treatment frequencies. It was found that this variation could largely be eliminated by subculturing all strains in M63 medium supplemented only with 1mM $MgSO_4$ such that growth of the cells during subculture was restricted.

2.2.6. Method for the Increasing of *E. coli* Cell Wall Permeability by EDTA Treatment.

The permeability of *E. coli* to large molecules such as actinomycin D was increased using an adaptation of the method of Lieve (1965).

Cells were grown o/n by the usual method and precipitated by centrifugation. Precipitated cells were washed once in 10mM Tris-Cl, pH 8 and resuspended in an equal volume of 33mM Tris-Cl pH 8. The resuspension was divided into two equal portions in sterile conical flasks. EDTA was added to one flask to 1mM. The flasks were incubated at 30°C for 10mins.

To test for the permeability of the cell wall 100μl aliquots of the EDTA treated and untreated cultures were plated, in top agar, onto LB agar plates. after setting of the top agar, a 0.6mm Whatman AA disc, inoculated with 10mg of actinomycin A (in aqueous solution) was placed into the centre of the plate and gently pressed in, taking care not to dislodge the top agar sideways. The plates were incubated inverted at 30°C o/n. The EDTA treated cell lawn showed a large zone (>1cm) of growth inhibition around the disc.

whereas the untreated cells grew unaffected right up to the disc.

EDTA treated cells were used, directly after treatment, in plate incorporation type assays (method 2.2.2.) for testing the transposogenicity of chemicals.

2.2.7. Protocol for the Estimation of Transposition Frequencies using the Mating Out Procedure with UWE110(pPH1J1) or UWE103(pPH1J1) and FE20.

The protocol for the subculture procedure, (2.2.4.), was followed up to the dilution steps. Rather than dilution and plating out directly, 1ml of each washed subculture was added to 1ml of FE20 cells (prepared by culturing from frozen permanents in LB medium supplemented with 20μg/ml nalidixic acid o/n at 30°C, 200rpm, pelleting by microcentrifugation and resuspending in sterile M63 medium). The cells were mixed gently and 1ml of the mixture applied to a sterile 0.22μm nitrocellulose filter on a Millipore glass filter holder. Liquid was removed under vacuum and the filter applied, face-up, to a fresh LB agar plate. After overnight incubation at 37°C, the cells were resuspended in M63 medium and diluted in ten-fold steps to 10^5 fold.

The number of FE20 cells acquiring the plasmid was estimated by plating 100μl aliquots of the 10^5 and 10^4 dilutions onto LB agar plates supplemented with 20μg/ml nalidixic acid and 20μg/ml gentamycin. Transposition frequency of Tn5 into the plasmid was estimated by plating 100μl aliquots of the 10^1 and 10^2 dilutions onto the same plates further supplemented with 50μg/ml kanamycin. All platings were again performed in triplicate.

Where required, the survival of UWE103/UWE110(pPH1J1)

could be estimated by plating 100μl aliquots of the 10^5 dilution onto LB/50μg/ml kanamycin plates (the number of colonies arising from occasional FE20(pPH1J1::Tn5) cells is assumed to be insignificant at this dilution).

2.2.8. Protocol for the Transduction of Cells with Mud1(Apr, *lac*) using the MAL103 Lysogen.

This method was derived from that used in the original construction of MAL103 (Casadaban and Cohen, 1979).

Cultures of the Mu lysogen MAL103 and the recipient strain were grown overnight on LB medium, 30°C, 200rpm. After o/n growth (≈18 hours) both cultures were subcultured, under these same conditions (4ml into 50 for MAL103 and 3ml into 50 for the recipient) into fresh LB medium supplemented with 10mM $MgSO_4$/5mM $CaCl_2$ (to aid phage adsorption later). After the MAL103 subculture had reached an $OD_{(450)}$ of 0.9 to 1.2 (≈2.5 hours) it was transferred to a 45°C shaking water bath for 25 minutes to induce the Mu cts62/Mud1 phage. The subculture was then transferred to 37°C for one hour to allow the phage to develop and lyse the cells. The phage were harvested by the addition of 0.5ml chloroform to complete cell lysis, centrifugation at 12000g, 5°C for 20 mins and removal of the top 5ml of supernatant.

The recipient strain was harvested from the subculture by centrifugation, and resuspended in 1/5th the original volume LB medium. 0.5ml aliquots of the recipient culture were incubated with 0, 0.1, 0.2, 0.3, 0.4 and 0.5ml of lysate at room temperature (20±2°C) for 20 mins to allow adsorption and integration of the phage. The tubes were then diluted 10 fold and incubated at 30°C for 1 hour to allow expression of the Ap^r marker, and plated

in 100μl aliquots onto MacConkey lactose/25μg/ml ampicillin plates. The plates were incubated o/n at 30°C. Isolated white (*lac*$^-$) colonies were picked for further characterisation.

2.2.9. Test for the Presence of the Mu cts62 Helper Phage in Mud1 Lysogens Constructed from MAL103 Lysates.

Potential Mud1 lysogens were screened for the presence of the Mu cts62 helper phage, required for cell lysis of induced lysogens (ie. containing genes from the S end of the ß region, deleted in Mud1). Colonies were streaked onto two LB/50μg/ml ampicillin plates, one of which was incubated o/n at 30°C, the other at 42°C, along with MAL103 and the non-transduced recipient strain as positive and negative controls. Colonies which grew well at both temperatures were assumed to contain Mud1 only.

2.2.10. Test for the Heat Inducibility of Mud1 Lysogens by Incubation at 37°C.

Mud1 lysogens were screened for active transposition by streaking isolated colonies onto MacConkey lactose agar plates and incubating at 30 and 37°C. Growth of Mud1 lysogens at 37°C inactivates the temperature sensitive Mud1 repressor, allowing the prophage to enter the replicative transposition phase of growth. Lysogens grown at 37°C will therefore have a high proportion (up to 100%) of *lac*$^+$ colonies (appearing red on MacConkey lactose medium). Streaks which showed low frequencies of *lac*$^+$ colonies at 30°C and a high percentage of *lac*$^+$ colonies at 37°C were selected for further study.

2.2.11. Transduction/Transformation of Bacterial Strains with Mud1 on the Self-Transmissable Plasmid pPH1J1.

The Mud1 lysogen MC4100 Mud1(Apr, *lac*) was transformed with the self-transmissable plasmid pPH1J1 by filter mating of the recipient strain with UWE110(pPH1J1) using the procedure in section 2.2.7. After resuspension of the mating mixture, MC4100 Mud1(Apr, *lac*)(pPH1J1) transformants were selected by streaking a loopful of resuspension onto an M63 glucose plate supplemented with 25μg/ml ampicillin and 20μg/ml gentamycin. Transformant colonies were picked and grown up in LB/ampicillin/gentamycin medium o/n 30°C, 200rpm. After o/n growth cultures were transferred to 37°C for 3-5 hours to induce Mud1 transposition and increase the likelihood of the plasmid obtaining a copy of Mud1. The transformant was then filter mated with a culture of FE20 using the same procedure as above. FE20(pPH1J1::Mud1) was selected for by streaking on MacConkey lactose/gentamycin/ampicillin/nalidixic acid agar plates. White colonies arising after overnight incubation at 30°C were screened for Mud1 induction at 37°C as in section 2.2.10. FE20 cells which had inherited an active Mud1 prophage on the plasmid were subsequently used to introduce Mud1 into other bacterial strains, using the multiple amino acid requirements of FE20 as a counter selecting marker after filter mating.

2.2.12. Transduction of UWE110(pPH1J1) to *recA*- using the Transducing Bacteriophage T4GT7.

This method is adapted from Plakidou *et al* (1984).

An overnight culture of UWE110(pPH1J1) in LB/kanamycin/gentamycin was subcultured (1 in 5) into 20ml of the same

medium. After the cells had reached mid log phase (OD_{600}=0.8) the cells were pelleted by centrifugation and resuspended in 2ml fresh LB supplemented with 20μg/ml tryptophan. 0.1ml Aliquots of the resuspension were incubated with 0.1, 0.2, 0.3, 0.4 and 0.5ml of a T4GT7 stock containing 10^9 plaque forming units (pfu) ml^{-1}. The T4GT7 stock, a gift from Dr. G.Salmond (Dept. Biol. Sci.), was prepared from a phage lysate of *E. coli* strain JC10240 which contains the tetracycline resistance carrying transposon Tn10 inserted near to a *rec*A marker. After incubation at room temperature for 20mins, 100μl of each mixture was spread onto LB/25μg/ml tetracycline agar plates. 100μl Of the phage stock and of untransformed UWE110(pPH1J1) were also plated as controls. After o/n incubation of the plates, individual colonies of Tc^r transductants were picked and tested for *recA* co-transduction (N.B. smaller colonies were picked preferentially as these were thought more likely to be *recA*-).

2.2.13. Test for the *rec* Status of T4GT7Tn10::*recA* Transduced Cells.

The *recA* marker causes cells to become more sensitive to the lethal and mutagenic effects of DNA damaging agents. In order to test for the *rec*A status of transformed cells, Tc^r colonies were streaked onto LB/tetracycline/gentamycin/kanamycin agar plates. Individual colonies were picked and streaked onto plates containing 0, 3, 5 and 10μg/ml of the photo-sensitising, DNA binding compound nitrofurantoin (NF). Colonies of JC10240 and untransformed UWE110(pPH1J1) were also streaked as *recA* positive and negative controls. *recA*$^+$ Cells grew well at all NF concentrations whereas *recA*$^-$ cells failed to show any growth at 5μg/ml NF.

2.2.14. Improved Test for the *rec* Status of T4GT7Tn10::*recA* Transduced Cells.

An improved method for testing the *recA* status of transformed cells, avoiding the use of the carcinogen NF was devised taking advantage of the high sensitivity of *recA* cells towards UV light. Single colonies of transductants and *recA* positive and negative controls were streaked in straight lines onto 3 LB agar plates. The lids of the plates were left off and half of each plate covered with a piece of cardboard. The plates were then exposed to 254nm UV light from two Phillips TL 8W/08 bulbs at a distance of ~20cm for 30, 60 and 90 seconds respectively. *recA*- Cells showed inhibition of growth in the half of the plate exposed to the UV light after 30secs exposure, whereas *recA*+ cells grew well across the whole plate after 90secs exposure.

2.2.15. Transduction of *S. typhimurium* Strains with Mud1 using Lysates of KC89, MH3823, MH3824 or TL154.

It was discovered that *S. typhimurium* could not be transduced with lysates of MAL103, as the infectious host range of Mud1/Mu cts62 co-lysates did not include this bacterium although Mu is viable in *S. typhimurium* with the same orientation of the G segment (G+) as in *E. coli*. Additionally, it was found impossible to introduce Mud1 into *S. typhimurium* strains using the plasmid pPH1J1, although transformation of *S. typhimurium* with the native plasmid was readily achieved.

To overcome these problems the method of Csonka *et al* (1981), employing lysogens of Mud1 and a hybrid Mu cts62 helper phage containing the tail segment of the phage P1, conferring onto lysates the host range of P1

(which includes *Salmonella*) were used. Lysate preparation and transduction etc. were performed in an identical manner as that used for MAL103 lysates (sections 2.2.8. to 2.2.11.).

2.2.16. Protocol for *Salmonella* Mutagenicity/Transposogenicity Co-Tests using Ames Tester Strains Transduced with Mud1(Ap^r, *lac*).

It was not desirable to employ the standard Ames test protocol of Maron and Ames (1983) for mutagenicity assays when performing transposition/mutagenicity co-assays using Ames tester strains transduced with Mud1(Ap^r, *lac*). This was due to the presence of citrate in the Vogel-Bonner agar plates recommended. *lac*$^+$ Mud1 Lysates of *Salmonella* can grow using citrate as the sole carbon source in the absence of glucose, making Vogel-Bonner plates unsuitable for Mud1 transposition assays and necessitating two different types of medium for the co-assays.

In order to overcome this problem an adaption of method 2.2.2. using M63 lactose agar plates was employed. The cells were grown o/n, at 30°C, 200rpm in 25ml M63 medium supplemented with 5mM histidine, 0.1mM biotin. Cells were pelleted by centrifugation (12000g, 20mins, 5°C) and resuspended in fresh M63 medium (minus glucose). Cells were subsequently diluted and plated with and without test chemical as in 2.2.2 using supplemented top agar. Cell survival was measured using top agar containing, in addition to 0.05% glucose, 5mM histidine, 0.1mM biotin, the top agar used in estimating *his*$^+$ reversion frequencies contained 0.1mM histidine, 0.1mM biotin, 0.05% glucose, and that used for estimating transposition frequencies contained 5mM histidine, 0.1mM biotin.

2.2.17. Protocol for the Spot Testing of Chemicals in the Revised *Salmonella* Mutagenicity Assay.

100μl of an overnight culture of the appropriate tester strain were added to 2.7 ml top agar supplemented with 0.1mM histidine, 0.1mM biotin, 0.05% glucose and spread onto an M63 lactose agar plate. The top agar was allowed to set and a sterile Whatman AA disc, impregnated with 10μl of a solution of test chemical of an appropriate concentration, pressed gently into the centre of the agar. Care was taken not to displace the disc sideways on the plate.

The plate was inspected for the growth of a ring of *his+* revertant colonies around the disc after 48-72hrs incubation at 30°C.

2.2.18. Protocol for the Estimation of DEL and ICR Frequencies in *Saccharomyces cerevisiae* Strain RS112.

DEL And ICR frequencies in RS112 were measured using an adaption of the method of Schiestl (1989). An overnight culture of the yeast was grown by inoculating 25ml of SC-leu medium in a sterile 125ml conical flask with half of an isolated colony of the yeast from a fresh (<48hrs old) streak on a YPAD agar plate (the other half of the colony was streaked onto SC-his and SC-ade agar plates to check for a his , ade genotype). The flask was incubated o/n at 30°C, 200rpm. After overnight growth, the cell numbers were estimated by counting on a haemacytometer (1000× magnification) and the cells diluted with fresh SC-leu medium to 2×10^6 cells/ml. The subculture was added to Sterilin 25ml sterile, disposable universal vials in 10ml aliquots and incubated in the presence or absence of any test chemicals for 18hrs, 30°C, 300rpm. After the incubation period the cells were pelleted by

centrifugation in a bench top centrifuge (6000g, 5mins, room temp.). The cells were washed once in either 5% sodium thiosulphate (alkylating agents) or sterile distilled water (other chemicals) and once more in sterile distilled water before being resuspended in 10ml sterile 1mM EGTA. The cells were vortexed vigorously for 1 minute, counted using a haemacytometer (1000× magnification), repelleted and resuspended in 1ml sterile distilled water.

Serial 10-fold dilutions of each subculture were made to a final dilution factor of 10^{-4} of the original subculture (ie. 5 serial dilutions). 100μl aliquots of the 10^{-4}-fold dilutions were plated, in triplicate, onto SC plates, by mixing with 2.7ml sloppy top agar (kept molten at 47°C), mixed, poured and rapidly spread by tilting. After incubation at 30°C for 72hrs the colonies were counted and used to estimate the total number of viable cells in the subcultures.

HIS^+ and ADE^+ reversion frequencies were estimated by plating, in an identical manner, the 10^0 and 10^{-1}-fold dilutions onto SC-his and SC-ade plates (the actual dilutions used were altered, if necessary, to take into account high cell kill effects and/or high reversion rates post-treatment).

CHAPTER 3: STUDIES ON TRANSPOSITION IN THE TWO TESTER STRAINS MC4100 Mud1(Apr, *lac*) AND UWE103 (pBR322).

3.1. Investigations into a Possible Role for cGMP in Transposition.

3.1.1. The Effects of Membrane Permeable Derivatives of Cyclic Nucleotides on Transposition.

As was mentioned in 1.3 and 1.4, a possible role for cGMP in transposition has been postulated (Wilkins, 1987; Wilkins and Swoboda, 1987). Wilkins reported a rise in tf of both Mud1(Ap^r, *lac*) and Tn5 on treatment with dbcGMP, and a small decrease in tf of Mud1(Ap^r, *lac*) with dbcAMP, explained in terms of the 'yin-yang' hypothesis of cyclic nucleotide action (Goldberg *et al*, 1975). The two monobutyryl derivatives of cGMP, N^2 and O-monobutyryl cGMP (N-mbcGMP and O-mbcGMP) were also reported to have a transposogenic effect on Mud1(Ap^r, *lac*).

Initial experiments were performed in association with Gary Wilkins. They were designed to confirm the cyclic nucleotide observations with MC4100 Mud1(Ap^r, *lac*), and to extend the type of experiments used to include a subculture procedure so that the strains could be treated with the test chemical for a well defined period of time, in the presence of other factors (*e.g.* S9 mammalian metabolic system) which may interfere with the plate incorporation assays. It was also thought desirable to investigate any difference in effect of the cyclic nucleotides between temperatures at which the Mud1(Ap^r, *lac*) temperature sensitive repressor was active (30°C) and inactivated (37°C), as any effect of the nucleotides on the repressor may thus become apparent.

Four cyclic nucleotide derivatives were tested in a subculture type experiment following the procedure in 2.2.4, cells were incubated in the presence of 25μg/ml of the test chemical, for one hour, at either 30°C or 37°C.

The chemicals chosen were dbcGMP, dbcAMP and its monobutyryl derivative N^6-mbcAMP and N^6-monobutyryl inosine 3', 5'-cyclic monophosphate (mbcIMP), which was included as a control as it would be expected to have no effect on a cGMP/cAMP mediated system. The concentration of chemicals was chosen to be ≈ 10 times higher than that which gave a significant effect in the plate incorporation assays (*i.e.* 50μg/plate ≈ 2.5μg/ml assuming an even diffusion of chemical through the plate), hopefully overcoming any reduction in effect due to the shortened incubation period in the presence of chemical. The results are presented in tables 3.1 to 3.4. Results are given as observed transposition frequencies (tf) and relative tf's (rtf) to the minus chemical control ± the standard deviation (SD) between three plate counts. Rtf's at 30°C and 37°C are calculated separately.

Table 3.1. The effect of dbcGMP on the transposition frequency of Mud1(Ap^r, *lac*) in liquid subculture.

Temperature in °C	Baseline tf	rtf±SD	tf + 25μg/ml dbcGMP	rtf±SD
30	7.26×10^{-6}	1±0.04	7.28×10^{-6}	1.00±0.05
37	1.56×10^{-5}	1±0.05	1.91×10^{-5}	1.22±0.27

Table 3.2. The effect of dbcAMP on the transposition frequency of Mud1(Ap^r, *lac*) in liquid subculture.

Temperature in °C	Baseline tf	rtf±SD	tf + 25μg/ml dbcAMP	rtf±SD
30	3.13×10^{-6}	1±0.14	3.07×10^{-6}	0.98±0.14
37	3.52×10^{-5}	1±0.02	4.77×10^{-5}	1.26±0.09

Table 3.3. The effect of mbcAMP on the transposition frequency of Mud1(Apr, *lac*) in liquid subculture.

Temperature in °C	Baseline tf	rtf±SD	tf + 25μg/ml mbcAMP	rtf±SD
30	2.13x10-6	1±0.04	2.15x10-6	1.01±0.07
37	7.2x10-6	1±0.06	7.18x10-6	1.00±0.07

Table 3.4. The effect of mbclMP on the transposition frequency of Mud1(Apr, *lac*) in liquid subculture.

Temperature in °C	Baseline tf	rtf±SD	tf + 25μg/ml mbclMP	rtf±SD
30	3.77x10-6	1±0.03	3.96x10-6	1.05±0.11
37	1.05x10-5	1±0.05	1.16x10-5	1.10±0.06

The results of these experiments were disappointing. At 30°C, no difference in tfs of subcultures +/- chemical over the standard deviation in the colony counts was observed for any of the cyclic nucleotides. Some small increases in tfs of cultures at 37°C + test chemical were observed in the cases of dbcGMP, dbcAMP and mbclMP, however, only in the case of dbcAMP was the increase greater than the SD, and when the general increase of tf at 37°C compared to those at 30°C (> 10 fold in the dbcAMP experiment) was taken into consideration, the differences were considered insufficient to draw any conclusions. The effect of incubation at 37°C on tf of Mud1(Apr, *lac*), although predicted due to the known temperature sensitivity of the transposase repressor of the construct, was interesting and further investigations are reported later (3.2).

The relatively high SD's observed in some of the tf

determinations are most likely to be due to pipetting errors in the plating out step of the assay and problems in the accurate determination of viable cell numbers on M63-glucose agar plates (subsequently overcome, see next paragraph but one). As more familiarity with the procedures involved was gained, SD's became generally lower, however, the SD for any one rtf determination was generally found to be consistently between 4 and 10% of the mean rtf value, as would be predicted from the number of sources of experimental error involved in the procedures (dilution of cultures, addition of test chemical and cells to top agar, variations in the volume of agar in hand poured plates, *etc.*). This means that small changes in tf were not generally taken as proof of a positive effect, even where the change in tf's exceeded their SD. In general, a change in tf of a minimum of ≈ 50% associated with a low SD of the values (both baseline and treated tf), was considered indicative of some effect. As previously mentioned (1.4), a consistent doubling in observed effect is considered a minimum requirement for the classification of compounds as 'positive' in carcinogenicity testing.

It should be noted that, for the plate incorporation type of assay, the SD of tf determinations, is an SD between three (or more) separate determinations, as each plating of cells in the presence of a chemical may be considered a 'separate' experiment. For the subculture type of experiment, the SD is merely an indication of *intra*-experimental errors as treatment with the chemical occurs prior to plating out. SD's however are no indication of the reproducibility of an experiment and, unless otherwise stated, all the results presented are representative of the results of two or more separate repetitions.

It was considered possible that the lack of effect on transposition by dbcGMP or dbcAMP was due to the unsuitability of the

assay procedure used for this chemical (*i.e.* a longer exposure to the chemical may be required), and for further studies using this class of compounds it was decided to use the plate incorporation assay of Wilkins (1987) (2.2.1). Initial results using this procedure with the strain MC4100 Mud1(Ap^r, *lac*) were hampered by the problems of uneven spreading of top agar on plating and overgrowth of colonies on the viable cell count plates, leading to inaccurate determinations (underestimation) of cell numbers. These problems were overcome by the use of slightly more top agar for the plating out step and the determination of a minimum glucose concentration necessary to allow adequate growth of all viable cells into colonies large enough to easily count. The latter was achieved by a simple titration experiment, adding glucose in a range of concentrations to top agar, and plating cell suspensions onto M63 agar plates lacking glucose (the same lactose containing plates could then be used for both counting of viable cell numbers and tf estimation). This modified procedure (2.2.3) was used for all subsequent experiments employing Mud1(Ap^r, *lac*). The increased top agar volume was also used in the plate incorporation assay procedure for UWE103(pBR322) (2.2.4).

Over the next few months, many experiments involving the treatment of both transposition tester strains with the dibutyryl derivatives of cGMP and cAMP were carried out in an attempt to confirm a role for the cyclic nucleotides in transposition of the two transposons. Some of the experiments also employed the cGMP-phosphodiesterase inhibitor M&B 22948 (figure 3.1), which was reported to have a small inducing effect on, and

increase the effect of dbcGMP on, the tf of Mud1(Ap^r, *lac*). The results presented in tables 3.5-3.9 are representative of the results obtained during this period.

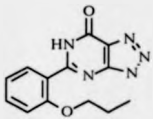


Figure 3.1. Chemical structure of the cGMP phosphodiesterase inhibitor M&B 22948.

Table 3.5. The effect of dbcGMP on the transposition frequency of Mud1(Ap^r, *lac*) in plate incorporation assays at 30°C.

dbcGMP in μg/plate	tf	rtf±SD
0	1.72x10-6	1.00±0.08
50	1.86x10-6	1.08±0.03
250	2.00x10-6	1.16±0.04
500	1.93x10-6	1.12±0.02

Table 3.6. The effect of dbcAMP on the transposition frequency of Mud1(Ap^r, *lac*) in plate incorporation assays at 30°C.

dbcAMP in μg/plate	tf	rtf±SD
0	1.74x 10-6	1.00±0.09
250	1.73x 10-6	0.99±0.10
500	1.82x 10-6	1.05±0.03

Table 3.7. The effect of dbcGMP in conjunction with 370μM M&B 22948 (concentration in top agar) on the transposition frequency of Mud1(Ap^r, *lac*) in plate incorporation assays.

dbcGMP in μg/plate	tf		rtf±SD	
	-M&B	+M&B	-M&B	+M&B
0	1.74x10-6	1.75x10-6	1.00±0.04	1.02±0.06
50	1.75x10-6	1.67x10-6	1.02±0.06	0.96±0.02

(M&B 22948 was added from a 15mM stock solution made up by dissolving the compound in a minimum amount of triethanolamine and making up to volume with sterile distilled water. The concentration used was the same as that previously reported to give potentiation of cGMP-mediated effects on Mud1(Ap^r, *lac*) transposition frequency (Wilkins, 1987)).

Table 3.8. The effect of dbcGMP on the transposition frequency of Tn5 in plate incorporation assays at 37°C.

dbcGMP in μg/plate	tf	rtf±SD
0	2.86x10-5	1.00±0.06
50	3.56x10-5	1.24±0.07
100	3.15x10-5	1.10±0.04

Table 3.9. The effect of dbcAMP on the transposition frequency of Tn5 in plate incorporation assays at 37°C.

dbcAMP in μg/plate	tf	rtf±SD
0	3.08x10-5	1.00±0.09
250	2.90x10-5	0.94±0.10
500	3.23x10-5	1.05±0.03

In experiments where M&B 22948 was used in plate incorporation assays with UWE103(pBR322), a significant and highly reproducible *decrease* in tf was observed in all estimations where M&B 22948 was included. As triethanolamine (TEA) had not been tested as a potential transposogen/anti-transposogen before its use as a solvent for other test chemicals, it was thought prudent to test its effects on transposition of both Mud1(Ap^r, *lac*) and Tn*5*. DMSO, although it had previously been tested and reported free of transposogenic effects (Wilkins, 1987) was also tested. The following results were obtained.

Table 3.10. The effects of DMSO and TEA on the transposition of Mud1(Ap^r, *lac*) in the plate incorporation assay.

Solvent concentration (in top agar)/mM	**tf**	**rtf±SD**
0	5.43×10^{-6}	1.00±0.02
(DMSO)		
261	5.48×10^{-6}	1.01±0.03
522	5.97×10^{-6}	1.10±0.02
(TEA)		
15.1	5.81×10^{-6}	1.07±0.07
75.7	5.53×10^{-6}	1.02±0.03
151	6.14×10^{-6}	1.13±0.03

Table 3.11. The effects of DMSO and TEA on the transposition frequency of Tn5 in the plate incorporation assay.

Solvent concentration (in top agar)/mM	tf	rtf±SD
0	1.42×10^{-5}	1.00±0.22
(DMSO)		
261	1.43×10^{-5}	1.01±0.03
522	1.42×10^{-5}	1.00±0.02
(TEA)		
6.95	3.41×10^{-6}	0.24±0.02
13.9	3.12×10^{-6}	0.22±0.05
69.5	7.10×10^{-7}	0.05±0.00
139	5.68×10^{-7}	0.04±0.01

The results of this test were quite dramatic, no effect on transposition of either transposon was observed with levels of DMSO higher than would generally be added to a test as solvent (522mM = 100μl added to top agar). There was also no effect of TEA on the transposition frequency of Mud1(Apr, *lac*) at equivalent amounts (139mM = 100μl added to top agar). There was however a very significant reduction in the tf of Tn5 (as measured in this assay) with relatively low levels of TEA (6.95mM =2.5μl added to top agar) (shown graphically in figure 3.2). This effect was not associated with any decrease in viability as viable cell counts at the highest dose were still 75% of the control counts and there was no decrease in viability at any of the lower doses. This would seem to rule out an inhibition of neomycin phosphotransferase or other cellular functions essential for cell survival on the

selection plates and may indicate a direct effect on either the Tn5 transposase or its repressor.

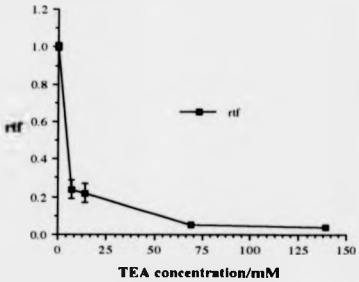


Figure 3.2. The effect of TEA on the transposition frequency of Tn5 in the plate incorporation assay.

Despite an extensive literature search, no convincing explanation for this effect was found.

The results of the previous experiments employing the cyclic nucleotide derivatives however, were less startling. No significant effect on transposition, in either strain, by either of the derivatives was observed. Occasional small increases or decreases in tf in excess of the SD were not reproducible between experiments. This was despite using concentrations of the derivatives upto 10× those previously employed (Wilkins, 1987). No effect was observed with the cGMP phosphodiesterase inhibitor M&B 22948.

These results would seem to argue against a role for cGMP in the

transposition of either Mud1(Ap^r, *lac*) or Tn5.

One final experiment was performed using dbcGMP. A literature search revealed a method for the increasing of *E. coli* cell wall permeability without significantly affecting cell viability (Leive, 1965). This was achieved by treating the cells with EDTA. This is thought to remove Ca^{2+} or Mg^{2+} ions from the lipopolysaccharide (LPS) and protein composed outer side of the outer bacterial membrane, thus disrupting the membrane structure, resulting in the replacement of LPS with phospholipid molecules, through which large lipophilic molecules may diffuse (Nikaido and Vaara, 1985). Thus treatment with EDTA creates cells analogous to the deep rough (*rfa*) mutants of *Salmonella typhimurium*.

Cells of MC4100 Mud1(Ap^r, *lac*) were treated with 1mM EDTA using method 2.2.6 and then used in the standard plate incorporation assay with dbcGMP. In this way it was hoped to overcome any permeability barrier presented by the cells to dbcGMP, a possible explanation for a lack of effect. The results, given in table 3.12, indicate that even in 'permeablised' cells, dbcGMP has no measurable effect on the transposition of Mud1(Ap^r, *lac*). EDTA treatment

Table 3.12. The effect of dbcGMP on the transposition of Mud1(Ap^r, *lac*) in *E. coli* cells with increased permeability to large molecules.

dbcGMP in μg/plate	tf	rtf±SD
0	1.84×10^{-5}	1.00±0.22
50	1.75×10^{-5}	0.95±0.12
100	2.08×10^{-5}	1.13±0.27

The unusually high apparent baseline tf and high SD's observed in this experiment were due to the determinations being based on low cell counts, following the dilution of overnight cultures during EDTA treatment. Low cell counts always produced over estimations of tf and high SD's. Where possible, plating densities for both systems were chosen so that the number of colonies on a plate was between 200 and 1000 after 72 hrs incubation, counts of < 50 colonies/plate were not generally used as the results obtained were unreliable.

3.1.2. The Effects of MNNG and Sodium Nitroprusside on the Tf of Mud1(Apr, *lac*) and Tn5.

A large transposogenic effect of the alkyl nitronitrosamines MNNG, ENNG and PNNG had previously been reported (wilkins, 1987; Wilkins and Swoboda, 1987). This had been attributed to a nitroxide radical (NO) mediated stimulation of guanylate cyclase activity. The effect was enhanced in the presence of M&B 22948 and decreased by the NO radical scavenger methylene blue. A small transposogenic effect was also noted for another guanylate cyclase stimulator, amyl nitrite, although no effect was seen for a third NO generator and alkylating agent, N-methyl-N-nitroso-p-toluene sulphonamide (MNTS).

It was decided to attempt to repeat some of the results obtained with MNNG, as a positive control for transposogenicity, and to investigate the effect of another potent NO generator shown to increase levels of cGMP in rat tissues, sodium nitroprusside (SNP) (Schultz, *et al*, 1977) on transposition in both strains.

MNNG was added to top agar from a stock solution of 1mg/ml, made up by diluting in the minimum amount of DMSO and making up in sterile distilled water. A range of concentrations of MNNG with and without

the inclusion of 370μM M&B 22948 were tested with MC4100 Mud1(Ap^r, *lac*) in plate incorporation assays and the following results obtained.

Table 3.13. The effect of MNNG, +/- 370μM M&B 22948, on the transposition of Mud1(Ap^r, *lac*).

MNNG in μg/plate	tf		rtf±SD	
	-M&B	+M&B	-M&B	+M&B
0	3.41×10-6	3.96×10-6	1.00±0.21	1.16±0.24
10	1.20×10-5	1.41×10-5	3.52±0.41	4.13±0.37
20	2.13×10-5	2.45×10-5	6.25±0.20	7.18±0.71
40	3.82×10-5	3.34×10-5	11.21±0.52	9.80±1.3

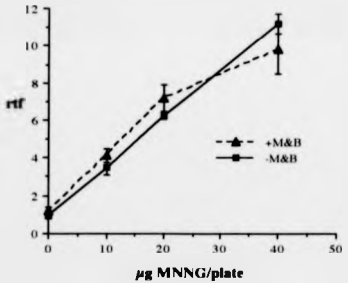


Figure 3.3. The effect of MNNG +/- M&B 22948 on the transposition of Mud1(Ap^r, *lac*).

The results indicate that MNNG does have a large transposogenic effect on Mud1(Ap^r, *lac*) with a linear increase in tf with increasing dose. M&B 22948 however, does not alter this effect over and above the SD of the samples. In similar experiments using UWE103(pBR322), the following results were obtained. (M&B was added as a solution in DMSO).

Table 3.14. the effect of MNNG, +/- M&B 22948, on the transposition of Tn5

MNNG in μg/plate	tf		rtf±SD	
	-M&B	+M&B	-M&B	+M&B
0	3.30x10-5	3.04x10-5	1.00±0.12	0.92±0.10
20	8.61x10-5	9.08x10-5	2.61±0.01	2.75±0.30
40	1.06x10-4	1.04x10-5	3.29±0.15	3.75±0.51
60	1.67x10-4	1.80x10-5	5.06±0.07	5.45±0.32

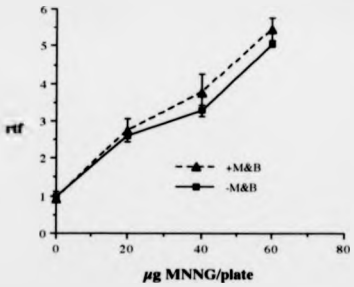


Figure 3.4. The effect of MNNG +/- M&B 22948 on the transposition of Tn5

Once again a large transposogenic effect of MNNG is evident. The lower levels rtf obtained in this experiment compared to the experiments with Mud1(Apr, *lac*), with sightly higher MNNG concentrations, probably reflect the extracellular reaction of MNNG with cysteine and other thiols in the complex LB medium used. M&B 22948 again had no significant effect on these results.

SNP was added, as an aqueous solution, to top agar, up to a concentration at which a 10-50 fold induction of cGMP levels was observed in rat ductus deferens (Schultz *et al*, 1977). The results, given below, indicate that, at the concentrations used, there was no significant induction of transposition of Mud1(Apr, *lac*). The experiment was not repeated with UWE103(pBR322). The use of higher doses was precluded by the onset of toxic effects at concentrations above 0.1M, with a resultant false depression of tf's.

Table 3.15. The effect of SNP on the transposition of Mud1(Apr, *lac*)

SNP in mM	tf	rtf±SD
0	6.76x 10-7	1.00±0.06
10	6.62x 10-7	0.98±0.08
50	7.37x 10-7	1.09±0.05
100	9.46x 10-7	1.14±0.02

The observation of transposogenicity of MNNG meant that it was the only chemical so far studied which could be used as a positive control in future experiments to increase the sensitivity of tester strains to transposogenic treatment, and the development of new tester strains. In preliminary experiments, the suitability of the subculture preincubation type of assay was investigated by incubating MC4100 Mud1(Apr, *lac*), for a range of

times, with and without MNNG (1μg/ml), using the method in 2.2.4. The following results were obtained.

Table 3.16. The effect of 1μg/ml MNNG on the transposition of Mud1(Ap^r, *lac*) in liquid subcultures.

Subculture time in hrs	tf		rtf±SD	
	-MNNG	+MNNG	-MNNG	+MNNG
0	2.15x10-6	-	1.00±0.16	-
3	2.37x10-6	4.62x10-6	1.10±0.04	2.15±0.15
5	1.77x10-6	4.31x10-6	0.82±0.03	2.00±0.04
7	1.62x10-6	4.21x10-5	0.75±0.04	1.96±0.19

(Rtf's were calculated relative to the 0hr control)

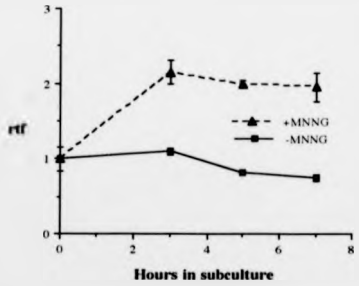


Figure 3.5. The effect of 1μg/ml MNNG on the transposition of Mud1(Ap^r, *lac*) in liquid subculture

The results indicate an approximate 2 fold increase in tf after 3 hours incubation with MNNG, and no further increases in tf after longer incubation. The apparent decrease in baseline tf over the course of the experiment was associated with an increase in cell numbers from 4.00×10^9 cells/ml to 5.88×10^9, this was due to the presence of glucose in the subculture medium. It was anticipated that the restriction of division during subculture may reduce the fluctuations in baseline tf and lead to more reproducibility in results, it was not known whether the induction of transposition by transposogens would be dependent on the presence of a carbon source in the medium. The reversion of *his* mutations by mutagens in *Salmonella typhimurium* is known to be more efficient when the cells are allowed to undergo 1-2 rounds of cell division in the presence of the mutagen by the addition of low levels of histidine to the test plates (Maron and Ames, 1983).

Cells were incubated in the presence and absence of 2μg/ml MNNG (concentration increased in order to achieve a higher observed effect), by the method in 2.2.5, omitting glucose from the subculture medium. The time of incubation was decreased to a minimum of 1 hour. The following results were obtained.

Table 3.17. The effect of 2μg/ml MNNG on the transposition of Mud1(Ap^r, *lac*) in liquid subcultures.

Subculture time in hrs	tf		rtf±SD	
	-MNNG	+MNNG	-MNNG	+MNNG
0	3.63×10^{-6}	-	1.00±0.07	-
1	4.87×10^{-6}	1.87×10^{-5}	1.34±0.07	5.15±0.28
3	-	2.12×10^{-5}	-	5.84±0.30
5	8.15×10^{-6}	2.23×10^{-5}	2.25±0.02	6.14±0.39

(Rtf's were calculated relative to the 0hr control)

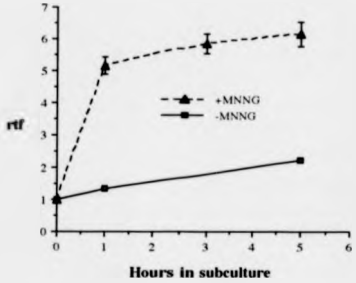


Figure 3.6. The effect of 2μg/ml MNNG on the transposition of MudI(Ap^r, *lac*) in liquid subculture.

The results again show an induction of transposition occurring before the first data point. Following this, a slight upwards drift in the tf of both treated and untreated cultures is observed, this is easily explained in terms of accumulated spontaneous transposition events during a period of zero growth (there is no change in viable cell numbers over the course of the assay). cell counts of the treated cultures are slightly lower than the untreated, 2.5×10^9/ml compared with 3×10^9/ml, indicating some cell kill. SD's of all data points are much improved in the second draft subculture assay and no requirement for glucose in the medium for the induction of transposition is observed, any energy requirement being supplied from intracellular pools (the possibility that the plateau in transposition induction is due to the exhaustion of these pools is eliminated by the observation of a greater induction in the second experiment relative to the first where a lower concentration of MNNG was used).

In an attempt to determine the onset of effect by MNNG, the above experiment was repeated with shorter incubation times.

Table 3.18. The effect of 2μg/ml MNNG on the transposition of Mud1(Apr, *lac*) in short-term liquid subcultures.

Subculture time in minutes	tf		rtf±SD	
	-MNNG	+MNNG	-MNNG	+MNNG
0	5.93x10-6	"	1.00±0.10	"
15	"	5.93x10-6	"	1.00±0.03
30	"	8.72x10-6	"	1.47±0.07
45	"	1.09x10-5	"	1.84±0.16
60	5.34x10-6	1.26x10-5	0.90±0.02	2.12±0.05
90	6.16x10-6	1.26x10-5	1.04±0.07	2.12±0.09

(Rtf's were calculated relative to the 0hr control)

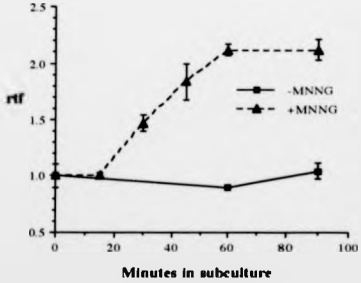


Figure 3.7. The effect of 2μg/ml MNNG on Mud1(Apr, *lac*) in short-term subcultures.

The results show that the induction of transposition of Mud1(Ap^r, *lac*) by MNNG occurs with after a lag phase of approximately 15 minutes, after which time the tf increases in an apparently linear fashion, reaching a maximum by 1 hour. This interpretation is necessarily an approximation due to the small number of data points used (limited by the physical and logistical necessities of the experiment, time taken for sample acquisition, number of dilutions, number of platings *etc.*). A much larger number of points would be expected to show a gradual rise in the rate of increase of tf following the lag phase, perhaps over several minutes, and a similar decrease as the plateau level was reached (the plateau presumably due to the exhaustion of the transposogenic substrate). The lag phase observed may indicate *de novo* protein expression in response to MNNG, necessary for its effect on transposition. This is probably the transposase (Mu A) protein but may be a protein responsible for derepression of Mu, possibly by proteolysis of the Mu repressor.

3.2. The Effect of Incubation at 37°C on the Transposition Frequency of Mud1(Ap^r, *lac*).

As previously mentioned, the Mud1 prophage has a temperature sensitive repressor function which leads to an uncontrolled level of replicative transposition when the host is incubated at an increased temperature. An apparent tf approaching 1.0 (ie 100% of viable cells containing Mud1 inserted next to an external promoter) was reported for tests involving incubation of the plates at 37°C (Wilkins, 1987). Inactivation of the phage repressor is a possible mechanism of transposogen action and it was thus decided to study the heat inactivation of repressor function in transposition assays involving incubation of the Mud1(Ap^r, *lac*) tester strain at 37°C for defined periods of time. Some increase in tf was already evident after 1 hour in subculture at 37°C (3.1.1) and initial experiments were performed, using the original

subculture procedure (2.2.4), to measure the level of tf increase after 0.5 to 7 hours incubation at 37°C. Cells were cooled rapidly on ice prior to pelleting and the washing steps were omitted.

Table 3.19. The effect of incubation at 37°C on the transposition of Mud1(Apr, *lac*).

Subculture time at 37°C in hours	tf	rtf±SD
0	$1.8 2 10^{-6}$	1.00±0.20
0.5	$1.8 2 10^{-6}$	1.00±0.02
1	$3.2 5 10^{-6}$	1.79±0.53
3	$7.7 7 10^{-6}$	4.27±0.64
5	$1.2 0 10^{-3}$	661±18.1
7	$3.8 8 10^{-3}$	2130±75.2

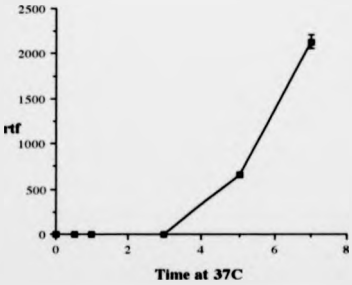


Figure 3.8. The effect of incubation at 37°C on the transposition of Mud1(Apr, *lac*).

The results show that, after a lag phase lasting approximately 30 minutes, transposition of MudI(Ap^r, *lac*) becomes derepressed resulting in a rapid and dramatic rise in tf over several hours. In several subsequent repeats of the above experiment, the results obtained were superficially similar, the 30 minute lag in effect was highly reproducible, as was the following rise in tf, however, the *amount* of increase in tf observed after a given period at 37°C was highly variable. The rtf after 5 hours at 37°C varied from 69.1 to 2330 in different experiments. This may be related to the baseline tf of the original overnight culture used (baseline tf 1.06×10^{-5} and 1.28×10^{-6} respectively for the above), however, this relationship is not absolute, and different subcultures from the same overnight also showed a high degree of variability. The slightly more reproducible subculture procedure (2.2.5) was tried, however, incubation at 37°C resulted in very high levels of cell death and no useful data was obtained. Cell death in the original subculture experiment was ~ 50% after 7 hours, probably due to a high number of inactivating Mu insertions in essential host genes. The high levels of kill in subcultures lacking glucose probably reflects a high energy requirement for such high levels of transposition.

The variability in heat induction meant that it was unfeasible to study the heat induction of MudI(Ap^r, *lac*) transposition in conjunction with any possible moderators of this effect. it had been suggested that the protease inhibitor antipain Phe-CO-Arg-Val-Arg-al), which has been shown to inhibit transformation *in vitro* (Kennedy and Little, 1978) and to reduce the frequency of chromosomal abnormalities in cells from patients suffering from Bloom's syndrome (Kennedy *et al*, 1984), may inhibit a possible protease

involvement in Mu repressor heat inactivation. Experiments to investigate this possibility proved to be impossible using these procedures. The overwhelming likelihood however, is that the temperature sensitivity is due to the physical instability of the mutant *c* gene product at the elevated temperature.

The lag phase in induction observed, again suggests that *de novo* protein synthesis is required. As previously mentioned, protein synthesis is known to be required for Mu transposition due to the stochiometric use of the transposase protein, and the lag phase probably reflects this requirement.

3.3. The Effects of Methyl Viologen (Paraquat) and Mitomycin C on the Transposition of Mud1(Apr, *lac*) and Tn5.

As it was felt desirable that any new test for agents which promote large scale alterations in chromosome structure should be able to detect the known clastogenic effects of raised intracellular levels of superoxide radicals (O_2^-, OH^-), it was decided to test the effects of two known generators of intracellular O_2^- on transposition frequencies in both tester strains. A more detailed discussion of the biological effects of paraquat (PQ) and mitomycin C (MC) is given in chapter 6.2.3.

Overall, the results were disappointing. Wilkins (1987) had reported an approximate 1.5 fold induction of transposition of Mud1(Apr, *lac*) in the presence of a crude system for the generation of *extracellular* O_2^- based on xanthine and xanthine oxidase, moreover, the effect was eliminated in the presence of superoxide dismutase. It was therefore anticipated that the generation of *intracellular* O_2^- by PQ in particular, would be highly transposogenic. The results are shown in tables 3.20-3.23.

Table 3.20. The effect of paraquat on the transposition of Mud1(Ap*r*, *lac*).

PQ concentration in top agar/μM	survivors in %	tf	rtf±SD
0	100	3.82x 10-6	1.00±0.07
5	100	3.59x 10-6	0.94±0.09
10	90	4.12x 10-6	1.08±0.11
100	53	4.78x 10-6	1.25±0.07
1000	0	-	-

Table 3.21. The effect of paraquat on the transposition of Tn5.

PQ concentration in top agar/μM	survivors in %	tf	rtf±SD
0	100	7.93x 10-5	1.00±0.05
5	100	8.48x 10-5	1.07±0.09
10	94	9.04x 10-5	1.08±0.11
100	65	1.36x 10-4	1.71±0.22
1000	0	-	-

Table 3.22. The effect of mitomycin C on the transposition of Mud1(Ap*r*, *lac*).

μg MC/plate	survivors in %	tf	rtf±SD
0	100	1.88x 10-6	1.00±0.07
0.25	78	2.41x 10-6	1.28±0.08
1.25	51	*1.63x 10-6	*0.87±0.02
2.5	15	5.64x 10-7	0.30±0.02
20	0	-	-

* Although viable cell counts indicated a good survival rate, a large proportion of colonies were of very small size, indicating a significant toxic effect.

Table 3.23. The effect of mitomycin C on the transposition of Tn5.

µg MC/plate	survivors in %	tf	rtf±SD
0	100	1.9×10^{-5}	1.00±0.05
0.25	80	2.48×10^{-5}	1.31±0.18
1.25	70	*2.13×10^{-5}	*1.12±0.07
2.5	57	*7.89×10^{-6}	*0.41±0.25
20	0	-	-

* Although viable cell counts indicated a good survival rate, a large proportion of colonies were of very small size, indicating a significant toxic effect.

Neither PQ or MC caused any large elevations in tf, some small positive effects were observed with both chemicals at dose levels where a significant toxic effect was first evident. The results indicate that both superoxide generating chemicals are weakly transposogenic but would suggest that neither of the tester strains can efficiently detect possible mutagens which may act by the production of superoxide radicals.

CHAPTER 4: DEVELOPMENT OF A MATING OUT ASSAY FOR TRANSPOSITION OF Tn5.

4.1. Rationale behind the Development of a New System for the Measurement of Transposition Frequencies.

As mentioned previously (1.4), one of the short-term aims of this project was the development of improved assay techniques allowing better quantification of transposition frequencies and, hopefully, showing greater sensitivity towards transposogenic treatments. Several aspects of the two existing assay systems were thought in need of improvement.

Firstly, the measurement of either *lac*⁻ reversion or of increased resistance to kanamycin was not a direct measurement of transposition. Both systems were subject to interference, apparently *lac*+ colonies may arise from *lac*⁻ cells growing on cell debris, an effect noticeable when very high densities of *lac*⁻ cells are plated onto lactose minimal medium plates, and resistance to high levels of Km may arise due to mutations in the *npt* gene promoter. Resistance of Km^r cells of the bacterium *Caulobacter* to 1mg/ml kanamycin has also been observed in cells grown on minimal media (Hodgson, D.A., personal communication), the same observation was also made with UWE103 on M63 minimal medium plates (Wilkins, 1987) and does not indicate a high *npt* copy number. Cells deficient in the genes for methylation of adenine and cytosine bases of DNA (*dam*⁻, *dcm*⁻) are also constitutively resistant to Km at the higher level (this work), presumably due to higher *npt* promoter activity in these strains.

Secondly, the events used to score transposition frequencies were not *absolute*. That is, cells were not always either '*lac*+ or *lac*⁻', or 'resistant or sensitive' to high levels of Km, a degree of variation was observed, such that a number of very small and intermediate sized colonies were apparent on lactose and 1mg/ml Km plates and accurate quantification of

the number of events was not always easy.

Thirdly, the two systems measured different 'end points', and, as such, were not directly comparable in the ff's measured.

Following discussions with Dr. D.A. Hodgson in the department of Biological Sciences, University of Warwick, a strategy to overcome these difficulties was developed. A new system capable of measuring transposition of (initially) Tn5 directly, adaptable for later use with Mud1(Ap*r*, *lac*), was devised. This was based on transposition into a target plasmid, followed by detection of this event by transformation of a second bacterial strain, sensitive to the antibiotic to which the plasmid encoded resistance, with the target plasmid and selection for transformants expressing transposon-encoded resistance.

4.2. Construction and Characterisation of the New Tester System.

4.2.1. Construction.

The strain of bacterium used in construction of the original Tn5 tester system, UWE103, and a second Tn5 carrying strain, UWE110, were transformed in the labs of Dr. D.A. Hodgson, with the self-transmissable plasmid (pPH1J1) by the filter mating method described in 2.2.7, from a multiply marked donor strain (J5-3(pPH1J1)). UWE103(pPH1J1) was isolated by selection on glucose minimal medium plates supplemented with 20μg/ml gentamycin (Gm), 50μg/ml Km. UWE110(pPH1J1) was isolated in the same way on plates further supplemented with threonine, leucine and thiamine.

The test was to involve the mating of the transposition tester strains with a second strain of *E. coli*, FE20, which encodes resistance to the

antibiotic nalidixic acid (Nal). Both the total number of FE20 cells inheriting the plasmid after mating, and the number inheriting both the plasmid *and* the transposon, could subsequently be easily quantified, in the presence of the donor strain, by selection on LB agar plates supplemented with Nal and Gm (FE20(pPH1J1)) or with Nal, Gm and Km (FE20(pPH1J1)::Tn5). The ratio of the two types of plasmid inherited would then be a direct measure of the frequency of transposition of the transposon into the plasmid (assuming that native and recombinant plasmids transfer at the same efficiency).

As a preliminary control, all three strains of cell were streaked onto LB agar plates supplemented with A: Km+Gm, B: Nal, C: Nal+Gm, D: Nal+Gm+Km. After overnight incubation at 37°C both UWE103(pPH1J1) and UWE110(pPH1J1) grew well on A but not on any of the others and FE20 grew well on B and none of the others. Thus the antibiotic resistance of the donor strain could be used as counter selection against the recipient and *vice versa*. Moreover, resistance to Gm and Nal, and Gm, Nal *and* Km could only be expressed by FE20 acquiring (pPH1J1) and (pPH1J1)::Tn5 respectively (Nal resistance is chromosomally encoded and therefore not transferable by any statistically important mechanism).

4.2.2. Characterisation and Confirmation of Responsiveness to Transposogens.

Three individual colonies each of UWE110(pPH1J1) and UWE103(pPH1J1) were picked from streaks on LB/Km/Gm plates and grown overnight in LB/Km/Gm medium at 37°C. 1ml of each culture was removed, pelleted in a microcentrifuge and resuspended in 1ml of fresh LB. Each was mixed aseptically with 1ml of an overnight culture of FE20 in LB/Nal medium

treated in the same way. The mixtures were applied to sterile 0.2μ nitrocellulose filters and the liquid removed by application of a vacuum. The filters were placed, face up, onto fresh LB agar plates and incubated overnight at 37°C to allow transfer of (pPH1J1) to FE20. The mixtures of cells were each resuspended in 1ml M63 medium and diluted in 10× steps to a dilution of 10^6 fold. 3× 100μl of each 10^5 and 10^4 fold dilution was plated, in top agar, onto LB/Nal/Gm plates to give the number of FE20(pPH1J1) colonies. Similarly, 3× 100μl of the 10^1 and 10^2 dilutions was plated onto LB/Nal/Gm/Km to give the number of cells acquiring both plasmid and transposon. The following transposition frequencies were measured.

Table 4.1. Frequencies obtained for Tn5 transposition into (pPH1J1) from the two donor strains.

Strain	tf
UWE103(pPH1J1)	
1	1.27×10^{-5}
2	1.24×10^{-3}
3	3.74×10^{-5}
UWE110(pPH1J1)	
1	1.84×10^{-5}
2	2.35×10^{-5}
3	5.67×10^{-5}

The tf obtained in each case (except UWE103(pPH1J1) isolate 2) was comparable to baseline tf's obtained in the UWE103(pBR322) tester strains, thus justifying the use of increased Km resistance as a measurement of

tf. The anomalous result obtained with the second UWE103(pPH1J1) isolate was probably due to the colony picked consisting of a large sub-population of cells with Tn5 insertions in the plasmid, due to a rare transposition event occurring early in colony growth. The first isolate in each case was selected and used in further studies.

The next step was to investigate whether the new assay also detected the one strong transposogen so far discovered. Overnight cultures of both donor strain were subcultured, following procedure 2.2.5, into six flasks. To three of each set of flasks, MNNG was added at a concentration of 3μg/ml. The flasks were incubated at 37°C for two hours and tf's of each subculture measured using the above procedure (2.2.7).

Table 4.2. Responsiveness of the two donor strains to treatment with MNNG.

Strain	tf		rtf±SD*	
UWE103(pPH1J1)	-MNNG	+MNNG	-MNNG	+MNNG
	1.19x10-5	6.69x10-5		
	1.22x10-5	6.14x10-5	1.00±0.02	5.54±0.39
	1.18x10-5	7.08x10-5		
UWE110(pPH1J1)	2.32x10-5	1.05x10-4		
	2.58x10-5	9.88x10-5	1.00±0.14	4.34±0.27
	1.96x10-5	9.25x10-5		

* Rtf's are calculated from the average tf of the three cultures with and without MNNG, SD's are the deviations between these values. The SD's of the individual tf determinations (*i.e.* the variation in colony counts) are not shown and are all < 5% of the mean value.

The results indicate that both strains are sensitive to tf induction by MNNG, UWE103 slightly more so than UWE110, although this may be due to differences in cell density between the two overnight cultures (not measured). The levels of tf induction obtained are comparable to those observed in equivalent experiments using UWE103(pBR322) indicating that the assay is not measurably different in sensitivity towards the transposogenic effect of MNNG.

The similar response of Tn5 to MNNG in both strains is not surprising as both are derivatives of *E. coli* K12, and may thus be expected to show the same behaviour. The similarity in response would also seem to rule out the inducibility of Tn5 by MNNG due to its insertion in a gene which has an altered expression in the presence of the mutagen, as the insertion site in both strains is different (maps between *metB* and *argE* in UWE110, insertion site not determined for UWE103).

The results also show excellent reproducibility between duplicate subcultures. In addition, the scoring of transposition events was much simplified by the 'absolute' nature of the end point *i.e.* recipient cells were either resistant to Km or sensitive to the drug, and all colonies grew to a comparable diameter, eliminating the possibility of inaccuracies due to the counting of false positives/negatives.

4.2. The Effects of *recA* and *dam*, *dcm* Mutants on the Transposition of Tn5.

UWE110(pPH1J1) was chosen to study the effects of two types of chromosomal mutation which may affect the tf of Tn5. UWE110(pPH1J1) was chosen, as a strain deficient in the enzymes responsible for host restriction modification, deoxyadenine methylase and deoxycytidine methylase (*dam*, *dcm*), was already available (GM48) which had originally been developed

from UWE110 and was thus identical apart from the two further mutations. It was thought desirable to test the effects of these mutations against an identical 'genetic background'.

4.2.1. The Effect of *dam*-, *dcm*- Mutations on the Transposition of Tn5.

Initially it had been wished to use the strain GM48 in plate incorporation type assays identical to those performed with UWE103(pBR322), this would be achieved by transforming the cells with pBR322 and measuring the frequency of resistance to 1mg/ml Km. In preliminary tests however, GM48 showed an already very high (≈ 100%) resistance to this concentration of antibiotic prior to transformation. There were two possible reasons for this observation (i) a very high frequency of tn5 transposition due to unregulated expression of the transposase gene, or (ii) enhanced expression of the *npt* gene.

In order to investigate further, GM48 was transformed with pPH1J1 using procedure 2.2.7. As a donor, FE20(pPH1J1) which had been screened for sensitivity to Km (*i.e.* was not carrying Tn5) was used as this could easily be counter-selected by plating the transformation mix on LB/Km/Gm plates. The low probability of *re*transformation of FE20 with pPH1J1::Tn5 was eliminated by screening transformants for Nal sensitivity.

Three transformants were picked and streaked onto LB/Km/Gm plates. One individual colony from each of these plates, and one colony from a further streak on LB(1mg/ml Km) (selected for high level kanamycin resistance, strain GM48H(pPH1J1)), were used to seed overnight cultures in LB/Km/Gm medium. An overnight culture of UWE110(pPH1J1) in the same medium was also grown. The five cultures were grown to an OD_{600} of between 1.0 and 1.1 and the tf of each culture measured using procedure 2.2.7.

Table 4.3. Comparison of Tf's of GM48(pPH1J1), UWE110(pPH1J1) and GM48H(pPH1J1).

Strain	tf	rtf±SD*
UWE110(pPH1J1)	3.15x10-5	1.00±0.06
GM48(pPH1J1)		
1	1.76x10-4	5.58±0.33
2	1.44x 10-3	45.7±8.50
3	4.32x10-4	13.7±1.13
GM48H(pPH1J1)	6.05x10-4	19.2±0.83

*Rtf's are calculated relative to the mother strain UWE110(pPH1J1).

The results show that transposition in the *dam*-, *dcm*- strains occurs at a frequency ~ 5-50 fold higher than in the *dam*+, *dcm*+, organism. The vastly greater tf suggested by the plating assay is false and probably due to increased *npt* expression in *dam*-, *dcm*- cells. The strain selected for increased resistance to Km shows a tf for Tn5 within the range of the other cultures, also ruling out high tf as the cause of increased Km resistance in GM48. The increased tf observed in the *dam*-, *dcm*- cells parallels the findings of Kleckner (1986) who reported a 10-100 fold increase in tf of Tn10 in a *dam*-, *dcm*- host and the later findings of Yin *et al* (1988) (published at about the time these results were obtained) who reported the regulation of Tn5, by *dam* methylation, at the transcriptional stage.

The sensitivity of Tn5 to the transposogenic effects of MNNG in a *dam*-, *dcm*- host was tested by using the third GM48(pPH1J1) isolate, which showed an intermediate amplification of baseline tf in the first experiment, in a subculture type experiment (2.2.7) +/- 3μg MNNG/ml for 2 hours at 37°C. UWE110(pPH1J1) was tested in parallel.

Table 4.4. Comparison of the effect of 3μg/ml MNNG on transposition in GM48(pPH1J1) and UWE110(pPH1J1) in liquid subculture.

Strain	tf		rtf±SD*	
	-MNNG	+MNNG	-MNNG	+MNNG
UWE110(pPH1J1)	2.13×10^{-5}	1.30×10^{-4}	1.00±0.12	6.39±0.69
GM48(pPH1J1)	2.14×10^{-4}	2.29×10^{-3}	1.00±0.23	10.70±1.34

*Rtf's are calculated relative to the -MNNG control in each case.

Along with the expected increase in baseline tf (10 fold in this case), a slightly higher sensitivity to the transposogenic effect of MNNG is also apparent in the *dam*, *dcm* host, although this effect is too small to draw any firm conclusions without repetition of the observation with other GM48(pPH1J1) isolates.

4.2.2. The Effect of a *recA*- Mutation on the Transposition of Tn5 in UWE110(pPH1J1).

The *recA* gene of *E. coli* is at the heart of the SOS regulatory system which controls the cell's responses to the potentially lethal effects of DNA damage and alterations to cellular metabolism (figure 4.1). Cleavage of the lexA repressor protein by active recA protease leads to the induction of 9 or more other SOS functions, the system can exist in various states of activation with some or all of the SOS genes repressed (Little, 1983). The induction of several prophages, including lambda, is known to involve the recA protein and to investigate whether the SOS response is involved in the induction of transposition by transposogens, perhaps by cleavage of transposon repressors, the construction of *recA*- mutants of transposition tester strains was undertaken.

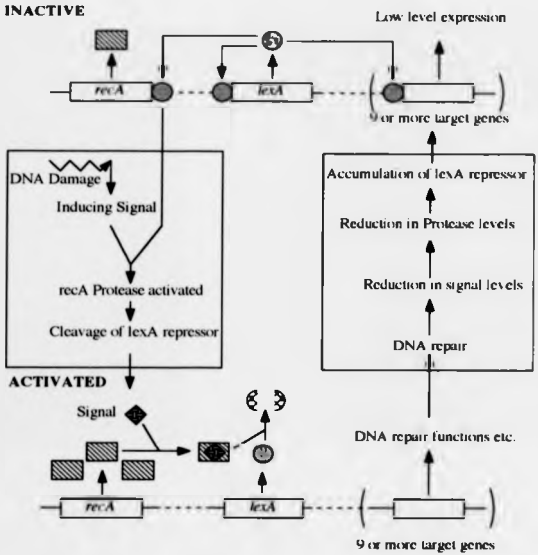


Figure. 4.1. Schematic representation of the SOS regulatory system. The inactive state (top) is normal in exponentially growing cultures. Transition to the active state (bottom) occurs in response to DNA damage (or other stimuli) and results in derepressed expression of SOS genes following inactivation of the lexA repressor by an activated recA protease. A return to the inactive state occurs when the level of signal reduces, following the action of SOS functions, and the lexA protein levels can again accumulate.

UWE110(pPH1J1) was transformed to *recA*⁻ by the procedure of Plakidou *et al* (1984) (2.2.12), employing a stock of the generalised transducing phage T4GT7 grown on *E. coli* JC10240, a *recA*⁻ strain with Tn10 (tetracycline resistance encoding) inserted next to the *recA* allele. *recA*⁻ Co-transduction with tetracycline resistance was screened for by methods 2.2.13 and 2.2.14, using JC10240 and *E. coli* K12 as *recA*⁻ and *recA*⁺ controls. *recA*⁻ Transductants were further screened for the *thr*, *leu* and *thi* markers of UWE110 as well as for Gm and Km resistance. One isolate, (UWE110 *recA*⁻ (pPH1J1) R1) was selected for further study on the basis of its high sensitivity towards nitrofurantoin and UV light (see 2.2.13/2.2.14).

In order to test the effects of MNNG on the *recA*⁻ tester strain, a subculture experiment was performed following procedure 2.2.5. UWE110 *recA*⁻ (pPH1J1) R1 was incubated for 1 hour at 37°C, with and without MNNG at 1, 2 and 3μg/ml. UWE110(pPH1J1) was also treated, in parallel, with 3μg/ml MNNG, as a positive control.

Table 4.5. Initial results obtained by subculture of UWE110 *recA*⁻ (pPH1J1) R1 +/- MNNG.

MNNG in μg/ml	tf	rtf±SD
0	3.42×10^{-5}	1.00±0.05
1	1.60×10^{-5}	0.49±0.04
2	1.18×10^{-5}	0.36±0.13
3	6.70×10^{-6}	0.20±0.02

This drop in rtf was unexpected, the rtf of the control (*recA*⁺) culture was 6.78±0.33 in the presence of 3μg/ml MNNG and the number of transformants (FE20(pPH1J1)) was approximately the same in each case(~ 9×10^{8}/ml). However, as it was expected that the *recA*⁻ strain would be

considerably more sensitive to the mutagenic and toxic effects of MNNG, the experiment was repeated with the inclusion of a plating of the 10^6 and 10^5 dilutions of the transformation mixture on LB/Km plates to give an estimation of UWE110 *recA*- (pPH1J1) R1 survival. The results showed a similar level of apparent depression of tf and also indicated that the percentage survival rates (compared to the untreated subculture) were 10, 7.5 and 4.4% for 1, 2 and 3μg/ml MNNG respectively, indicating a possible link between cell kill and apparent depression of tf. The high numbers of FE20(pPH1J1) transformants obtained reflects the efficiency of the mating out procedure, *i.e.* a small ratio of donor to recipient cells can still give rise to high transformation frequencies with the self-transmissable plasmid.

The effect of cell kill on tf was further studied by reproducing the kill effect in the *recA*+ strain UWE110(pPH1J1) by incubation of subcultures in the presence of up to 20μg/ml MNNG for 1 hour at 37°C.

Table 4.6. The effect of high levels of MNNG on the transposition of Tn5 in liquid subcultures of UWE110(pPH1J1).

MNNG in μg/ml	Survivors in %	tf	rtf±SD
0	100	2.05×10^{-5}	1.00±0.07
5	30.3	2.81×10^{-5}	1.36±0.17
10	4.1	1.08×10^{-5}	0.52±0.01
20	1.0	2.33×10^{-6}	0.11±0.02

The results showed that the cell kill related depression of tf was not specific to the *recA*- strain and that, at high levels of toxicity, no reliable data on transposogenicity can be obtained. Some residual transposogenic effect is observed at 5μg/ml MNNG where a comparatively moderate toxic effect is evident.

In a series of further subculture experiments, UWE110 *recA*- (pPH1J1) R1 was incubated with MNNG concentrations as low as 0.01μg/ml, to investigate whether sub-lethal levels of MNNG were transposogenic to the strain, as would be predicted if transposogenicity was related to mutagenicity (*i.e.* mutagenic DNA modifications inducing transposition). A subculture of UWE110(pPH1J1) +/- 2μg/ml MNNG was again included as a positive control. The results of the experiment presented in table 4.7 are representative of the observations.

Table 4.7. The effect of sub-lethal levels of MNNG on transposition in UWE110 *recA*- (pPH1J1) R1.

MNNG in μg/ml	Survivors in %	tf	rtf±SD
0	100	2.93x10-6	1.00±0.11
0.01	100	2.83x10-6	0.97±0.08
0.05	94.2	3.07x10-6	1.05±0.17
0.1	71.5	3.15x10-6	1.08±0.11
1	5.96	1.80x10-6	0.62±0.01

The results indicate that at levels of MNNG which are sub-lethal, or show only moderate levels of toxicicity, there is no induction of Tn5 transposition in UWE110 *recA*- (pPH1J1). At MNNG concentrations which show low levels of toxicicity in equivalent *recA*+ strains, a significant transposogenic effect is also observed. There are two possible reasons for this lack of effect in *recA*- cells. Firstly, transposogenicity of MNNG is dependent on the presence of an intact cellular SOS response, perhaps being mediated via recA protease activity. Secondly, the transposogenic effect is *epigenetic* and not linked to the mutagenic properties of the chemical, the lack of effect is thus due to an inadequate concentration of the transposogen. The second

explanation would also explain the lack of significant transposogenicity observed for other classes of mutagens, of similar mutagenic potency, tested.

Due to a lack of time available, the effect of the *recA* mutation on the transposition of MudI(Apr, *lac*), which was expected to yield a strain showing a similar lack of sensitivity towards the transposogenic effects of MNNG, was not studied.

CHAPTER 5: CONSTRUCTION OF TRANSPOSITION TESTER STRAINS IN *SALMONELLA TYPHIMURIUM* COMPATIBLE WITH THE AMES TEST.

5.1. Rationale Behind the Development of Transposition Tester Strains in *Salmonella typhimurium*.

The Ames *Salmonella* mutagenicity test is the most widely employed short-term test for the detection of mutagens and has been tested on very large numbers of chemicals. It has been estimated that the test detects about 83% of the known carcinogens tested, as mutagenic (Maron and Ames, 1983).

The test employs a series of strains of *S. typhimurium* LT2 containing mutations in the histidine operon (*i.e. his*). The particular mutation present determines the type of mutational event detected. Agents which cause base pair substitutions, including hard methylating agents such as MNNG, are detected by strains containing a proline to leucine (-GGG- to -GAG-) point mutation in the *hisG* gene (*hisG46*) (Barnes *et al.* 1982). Frameshift mutagens are detected in strains carrying the *hisD3052*, -1 frameshift mutation, with the repetitive sequence -CGCGCGCG- which acts as a 'hotspot' for base pair mismatches, stabilised by frameshift mutagens.

Several of the strains carry further mutations which improve the sensitivity of the strain towards mutational reversion. Two main mutations are used. The *rfa* or deep rough mutants are partially deficient in the lipopolysaccharide barrier coating the cell surface, this increases permeability of the cell to large molecules such as benzo(α)pyrene. The second mutation, *uvrB*, is a deletion in a gene coding for the DNA excision repair system, rendering cells with the *uvrB* mutation much more sensitive to a wide range of mutagens. The inclusion of an R-factor plasmid, pKM101, in some of the strains, also increases sensitivity towards certain mutagens due to the

enhancement of host-encoded, error-prone SOS repair system (Shanabruch and Walker, 1980).

During discussions with Dr. D.A. Hodgson it was noted that the host range of Mu includes *S. typhimurium* and that the organism possesses no *lac* operon (*i.e. S. typhimurium* has a *lac*⁺ wild-type). *S. typhimurium* would therefore be an ideal host for the study of Mud1(Apʳ, *lac*) prophage transposition. Transduction of *S. typhimurium* strains with Mud1(Apʳ, *lac*) was expected to be trivial. Mu infects *S. typhimurium* with the same G-segment orientation (G+) as for *E. coli* and, it was believed, the same procedure as previously employed in the construction of *E. coli* MC4100 Mud1(Apʳ, *lac*), the use of lysates of *E. coli* MAL103 (2.2.8) (Wilkins 1987), could be used to transduce *S. typhimurium* with the prophage.

The construction of transposition tester strains in existing Ames test strains was predicted to confer several benefits. Firstly, the new tester strain would take advantage of existing mutations in the bacteria, such as *uvrB* and *rfa*, thus potentially making the strains more sensitive to potential transposogens. Secondly, the new system would be capable of measuring genetic mutation (*his* reversion) and transposogenesis (acquisition of a *lac*⁺ phenotype) simultaneously, allowing direct comparisons between the two events to be made. Thirdly, the inclusion of a transposition based assay in the standard Ames test may allow the detection of certain carcinogens not detected by the standard Ames test.

Three Ames tester strains were chosen for initial studies. All three contained the *hisG46* mutation as it was anticipated that MNNG would initially be employed to confirm transposition inducibility (MNNG efficiently reverts the *hisG46* mutation (Maron and Ames, 1982)). The potential effects of

the *uvrB* mutation on Mud1(Apr, *lac*) transposition were unclear, and may have been disadvantageous, thus it was thought necessary to investigate transposition in a *uvrB* strain compared to a *uvrB*+ strain. The *rfa* mutation was expected to be beneficial as sensitivity to chemicals is increased by raising the *apparent* concentration by improved diffusion into the cell. TA1535 has both the *uvrB* and *rfa* mutations, TA1950 has only the *uvrB* mutation and TA1975 has only the *rfa* mutation in addition to *hisG46*.

TA1535 was kindly donated by Dr. R. Tye of the Severn Trent Water Authority testing station in Coventry. TA1950 and TA1975 were generously sent to us by Dr. B. Ames from the Dept. Biochemistry, University of California, USA.

5.2. Construction of Mud1(Apr, *lac*) Lysogens in the Ames *Salmonella* Tester Strains TA1535, TA1950 and TA1975.

5.2.1. The Use of Lysates of MAL103.

Overnight cultures of all three *Salmonella* strains were treated with lysates of the *E. coli* Mud1(Apr, *lac*)/Mu cts 62 lysogen MAL103, using the procedure described in 2.2.8. Despite several repetitions with fresh MAL103 lysates, no Apr transductants of any of the three strains were obtained using this procedure. In a control experiment, large numbers of Apr transductants were obtained in *E. coli* K12 wild-type cells using the same lysates, confirming the presence of infectious phage particles in the lysates.

A subsequent review of the literature revealed that we had overlooked the fact that although the host range of Mu does include *Salmonella typhimurium*, and stable lysogens of the phage can exist in this

organism, the *infectious* range of Mu does not include *Salmonella*, infectious Mu particles are unable to recognise surface antigens of the cell and thus cannot adsorb and infect the cell.

5.2.2. Attempted Transformation/Transduction with pPH1J1::Mud1(Ap^r, *lac*) Constructs.

Due to the ease of transfer of Tn5 encoded information between strains of *E. coli*, on the self transmissible plasmid pPH1J1 (chapter 4), it was decided to attempt a similar transfer of Mud1(Ap^r, *lac*) between *E. coli* and *S. typhimurium*. In a preliminary experiment to test the feasibility of this approach, cells from an overnight culture of TA1535 on LB were mated with cells of FE20(pPH1J1) using the procedure in 2.2.7. TA1535(pPH1J1) was selected for by growth on M63 agar supplemented with histidine, biotin and 20μg/ml gentamycin. Large numbers of transformants were obtained confirming that the plasmid could successfully transfer between the two organisms.

The next step was the mating of FE20(pPH1J1) with MC4100, to obtain MC4100 Mud1(Ap^r, *lac*)(pPH1J1), using the same procedure with selection on M63/Ap/Gm plates. Once again large numbers of transformants were obtained. As it was impossible to counter select against MC4100 in the presence of any of the *Salmonella* strains, a third step was necessary, involving the *retransformation* of FE20 (non-plasmid bearing) and selection for FE20(pPH1J1)::Mud1(Ap^r, *lac*) as a counter-selectable shuttle between the two organisms. To improve the chances of co-inheritance of the prophage with the plasmid, as the tf of Mud1(Ap^r, *lac*) into the plasmid was not known, a culture of MC4100 Mud1(Ap^r, *lac*)(pPH1J1), in LB/Gm/Ap, was transferred

to 37°C for 3-5 hours before mating with FE20. FE20(pPH1J1)::Mud1(Ap^r, *lac*) was selected by spreading 100μl of the resuspended mating mixture onto McConkey lactose/Nal/Gm/Ap plates. A large number of both white (*lac*-) and red (*lac*+) colonies were obtained. Several of the white colonies were picked and streak purified for further characterisation. As the strain was to be used solely as a shuttle for the recombinant plasmid, extensive characterisation was thought unnecessary. It was, however, necessary to ensure that the plasmid was carrying a 'transposition competent' copy of Mud1(Ap^r, *lac*). This was ensured by streaking a loopful of an overnight culture of FE20(pPH1J1)::Mud1(Ap^r, *lac*) in LB/Gm/Ap, on to two McConkey lactose plates, followed by incubation of the plates at 30 and 37°C overnight. Incubation at 30°C should result in a low number of red colonies appearing against a background of predominantly white (*lac*-) colonies, indicating a low level of spontaneous transposition. Plates at 37°C should show a very high proportion of red (*lac*+) colonies due to heat inactivation of the Mud1 cts repressor.

The FE20(pPH1J1)::Mud1(Ap^r, *lac*) construct showing, by eye, the lowest levels of spontaneous transposition along with good heat inducibility, was chosen as the donor strain for matings with the *Salmonella* strains. The three Ames tester strains were mated with FE20(pPH1J1)::Mud1(Ap^r, *lac*) using procedure 2.2.7, and potential *Salmonella* transformants selected for by spreading the mating resuspension onto M63 agar supplemented with histidine, biotin, 20μg/ml Gm, 25μg/ml Ap. Despite the proven ease of transfer of pPH1J1 between the two organisms, no transformants of *Salmonella* inheriting the prophage Mud1(Ap^r, *lac*) along with the plasmid were obtained using this method. In control experiments, *E.*

coli K12 was successfully transformed with the recombinant plasmid and inherited a transposition competent copy of Mud1(Apr, *lac*), the reason for the lack of success with *Salmonella* remains unclear.

5.2.3. The use of Lysates from Mud1(Apr, *lac*) Lysogens Containing the Hybrid Helper Phage Mu cts 62 hP1#1.

Mud1(Apr, *lac*) lysogens of *Salmonella typhimurium* strains have previously been reported (Csonka *et al*, 1981). These lysogens were constructed from lysates of *E. coli*, strains carrying Mud1(Apr, *lac*) and an engineered helper phage, derived from the Mu cts 62 phage by the addition of genes from the phage P1 (whose infectious range includes *S. typhimurium*) encoding the tail genes of P1 responsible for recognition of, and adhesion to, the host cell surface antigens. These foreign sequences conferred upon the Mud1/Mu cts 62 lysates the host range of P1.

E. coli strains TL154 and KC89, lysogenic for Mud1(Apr, *lac*) and Mu cts 62 hP1#1 were kindly donated by Dr. L Csonka of the Dept. Biol. Sci. Purdue University, USA. Strains MH3823 and MH3824, identical genotypically to KC89, were generously sent by Dr. M Howe, Dept. Microbiol. University of Tennesse, USA.

Using lysates of these strains (procedure 2.2.15) the three Ames tester strains were successfully transduced with Mud1(Apr, *lac*). Several *lac*- colonies of each *Salmonella* transductant were picked and screened for low level base line transposition, heat inducibility of the prophage (2.2.10) and the presence of the Mu cts 62 hP1#1 helper phage (2.2.9). Potential tester strains were then tested for the retention of Ames tester strain characteristics.

Histidine (all strains) and biotin (TA1535 and TA1950)

requirement was checked by streaking onto selective M63 plates.

The presence of the *uvrB* marker in TA1535 and TA1950 was tested in an identical fashion to that used for the identification of the *recA* marker in *E. coli* (2.2.14).

The presence of the *rfa* mutation was confirmed in TA1535 and TA1975 in a similar fashion to the conformation of actinomycin sensitivity in *E. coli* after EDTA treatment (2.2.6). A 5mm. sterile filter paper disc inoculated with 10μl of a 1mg/ml aqueous solution of crystal violet was applied to the centre of the seeded plate and a zone of growth inhibition (> 1cm) checked after overnight incubation at 37°C.

Sensitivity of all of the tester strains to *his* reversion by mutagens was confirmed using the spot test procedure (2.2.17) with a 5mm filter paper disc impregnated with 10μl of a sterile, 1mg/ml MNNG solution (in DMSO/distilled water). Sensitivity was confirmed by a zone of growth inhibition followed by a ring of colony growth around the disc. Spontaneous reversion frequencies were also checked to be within the usual ranges (Maron and Ames, 1982) by plating of 100μl aliquots of overnight cultures of the strains on M63 glucose/biotin plates as described in 2.2.16.

Transductant colonies fulfilling all of the above criteria were chosen for further study.

5.3. Preliminary Results Obtained in Studies using TA1950 Mud1(Apr, *lac*), and TA1975 Mud1(Apr, *lac*).

Construction of the *S. typhimurium* Mud1(Apr, *lac*) tester strains had taken longer than anticipated and time pressures dictated that studies using the new tester strains would be limited.

Initial experiments to test the possibility of measuring tf of Mud1(Apr, *lac*) in the strains, using the Vogel-Bonner medium recommended for use in the Ames test (Maron and Ames, 1982) showed that on Vogel-Bonner lactose plates (supplemented with histidine and biotin) all of the strains showed apparent tf's of 100%. This was due to the ability of the strains to utilise the citrate in the medium as a carbon source.

Spot tests using MNNG (2.2.17) showed, however, that *his* reversion could equally well be measured on M63 glucose plates and a protocol for the consecutive testing of mutagenicity and transposogenicity of chemicals on the strains was devised (2.2.16).

Eight isolates of TA1975 Mud1(Apr, *lac*) and five of TA1950 Mud1(Apr, *lac*) were selected on the basis of low baseline levels of transposition and reversion, coupled with good sensitivity to heat induction of Mud1(Apr, *lac*) and MNNG induced *his* reversion in spot tests.

Overnight cultures of these isolates in M63 glucose medium, supplemented with 5mM histidine, 0.1mM biotin were subjected to plate incorporation assays using procedure 2.2.16, on plates containing 0, 6 and 30μg MNNG (TA1975 Mud1(Apr, *lac*)) and 0, 1, 10 and 20μg MNNG (TA1950). The lower doses used for TA1950 were due to the expected increase in sensitivity to the lethal and mutagenic effects of MNNG, conferred by the *uvrB* mutation in the strain. Due to the number of strains tested, only one or two platings per dilution per MNNG concentration were used and the results were only used as a qualitative measure of MNNG transposogenicity to the strains. No measure of mutagenicity was made at this stage as *his* reversion frequencies at all of these doses was expected to be high.

None of the TA1975 Mud1(Apr, *lac*) strains showed any

significant increases in tf with the two MNNG doses tested, a significant cell kill effect, ~ 40-80% at the higher dose, was, however, observed indicating an increased sensitivity to the toxic effects of MNNG compared to MC4100 Mud1(Ap^r, *lac*), possibly due to the *rfa* mutation allowing greater penetration of the cell by the drug. One isolate, TA1975 Mud1(Ap^r, *lac*)#10, was chosen for further study on the basis of moderate toxicity (60% kill), small induction of tf at the higher MNNG concentration (rtf = 1.31) and intermediate baseline tf (5×10^{-6}). All of the TA1975 Mud1(Ap^r, *lac*) isolates exhibited a baseline tf in the range $1\text{-}7\times10^{-6}$ indicating that naturally occurring transposition of Mud1(Ap^r, *lac*) occurs at about the same frequency in *S. typhimurium* as in an *E. coli* host although in later experiments TA1975 Mud1(Ap^r, *lac*) exhibited baseline tf's as low as 2×10^{-7}.

A much larger variation in baseline tf of the TA1950 Mud1(Ap^r, *lac*) isolates was observed in this initial experiment, the lowest was 7×10^{-7} and the highest 7×10^{-5} (mean = $1.5\times10^{-5}\pm1.3\times10^{-5}$), however the only two tfs not in the range $1\text{-}7\times10^{-5}$ were estimated from very low (> 100/plate) colony counts, and the 'natural' tf of Mud1(Ap^r, *lac*) in TA1950 appears to be ~ 10^{-5}, slightly higher than *E. coli* lysogens or isogenic (apart from *uvrB*) strains of *S. typhimurium*. The *uvrB* mutation would therefore appear to have an *inducing* effect on the transposition of Mud1(Ap^r, *lac*), implicating the type of DNA adducts normally repaired by this excision repair pathway, *e.g.* pyrimidine dimers, as transposogenic. One isolate, TA1950 Mud1(Ap^r, *lac*)#7, was chosen for further study on the basis of a large apparent increase in tf in response to MNNG (rtf + 20μg MNNG = 28), although this effect was associated with a high level (98%) of cell kill and was thus thought questionable especially as

the tf estimation was based on colony counts from only two plates per dose. Two of the other isolates tested also showed smaller increases in tf (rtf = 2.17 and 1.76) at this dose level with a lower cell kill (80% and40% respectively). On the basis of this preliminary data, TA1950 Mud1(Ap^r, *lac*) therefore showed considerable promise as a transposition assay strain.

Following the initial experiments, the tf of TA1975 Mud1(Ap^r, *lac*) was measured in response to MNNG in plate incorporation type assays. Mutagenicity assays were performed simultaneously using procedure 2.16. The range of MNNG concentrations was chosen taking into account the increased sensitivity of the strain to its toxic effects previously mentioned.

Table 5.1. The effect of MNNG on transposition frequency in TA1975 Mud1(Ap^r, *lac*).

MNNG in µg/plate	Survivors in %	tf	rtf±SD
0	100	1.48×10^{-7}	1.00±0,08
0.5	100	1.77×10^{-7}	1.19±0.16
2.5	96	1.79×10^{-7}	1.21±0.1
5	98	2.09×10^{-7}	1.41±0.12
10	96	1.75×10^{-7}	1.19±0.13
25	74	1.37×10^{-7}	0.93±0.20

The results were, at first disappointing. Despite low to moderate levels of MNNG toxicity effect, only a small amplification in tf was observed, high SD's between counts, due to the low baseline tf resulting in small numbers of colonies per plate (~ 100), made the observed increases (maximum rtf = 1.41±0.12) even less convincing. The results of the mutagenicity tests, however, were as expected. The spontaneous reversion frequency was

1.41×10^8, an amplification of this frequency by 36 fold (±8) was observed on the 0.5μg MNNG plates, the number of revertants on the higher dose plates was too high to count.

During the course of the experiment it was noted that growth of *lac*+ colonies on the transposition frequency testing plates was relatively slow compared to an *E. coli lac*+ colony. In the light of this it was decided to repeat the counts on the 0-10μg MNNG lactose plates after an extra 24 hours incubation at 30°C (*i.e.* final counts were taken after 96 hours incubation instead of the usual 72 hours). the counts on the glucose viable cell count plates did not increase in this period.

Table 5.2. The effect of MNNG on transposition frequency in TA1975 Mud1(Apr, *lac*). Colony counts after 96 hours.

MNNG in μg/plate	tf	rtf
0	2.44×10^{-6}	1.00±0.021
0.5	3.82×10^{-6}	1.57±0.25
2.5	4.95×10^{-6}	2.03±0.06
5	5.86×10^{-6}	2.4±0.19
10	4.31×10^{-6}	1.77±0.07

The results, also shown graphically in figure 5.1, indicate that, allowing for the slower growth rate of *lac*+ colonies of TA1975 Mud1(Apr, *lac*), a significant increase in tf of the prophage is observed in response to MNNG treatment. The maximum level of increase observed is much lower than seen in *E. coli* Mud1 lysogens (~ 2 fold compared with ~10 fold). The slow growth rate of lactose fermenting colonies may be due to an inefficient expression of the *lac* genes in *Salmonella* compared with *E. coli* (codon usage) or, more likely, to inefficient operation of the *lacY* gene product, the

membrane bound lactose permease protein, in this strain, perhaps due to the membrane alterations caused by the *rfa* mutation. The slow growth also explains the relatively low tf's measured for this strain.

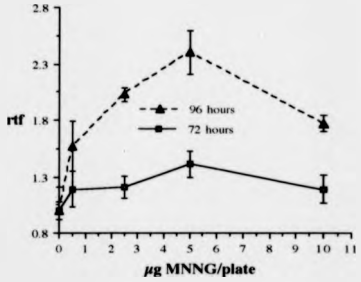


Figure 5.1. The effect of MNNG on the transposition of Mud1(Ap^r, *lac*) in TA1975. Colony counts after 72 and 96 hours.

In view of the observation of a high toxicity of MNNG to TA1950 Mud1(Apr, *lac*) in plate incorporation assays, it was considered that treatment with MNNG in subculture experiments, for short periods of time, may allow a better survival rate, *i.e.* removal of the tester strains from media containing MNNG after 2 hours may allow a higher percentage of the cells to recover and form viable colonies.

TA1950 Mud1(Apr, *lac*) was treated with a range of MNNG

concentrations in subculture, for 2 hours at 30°C, following procedure 2.2.5, followed by plating as in 2.2.16. A mutagenicity assay was also performed to check for the induction of *his* reversion by these short treatments.

Table 5.3. The effect of MNNG on the transposition in TA1950 Mud1(Ap^r, *lac*) liquid subcultures.

MNNG in μg/ml	survivors in %	tf	rtf
0	100	4.74×10^{-5}	1.00±0.01
1	100	4.86×10^{-5}	1.02±0.11
1.5	93	4.68×10^{-5}	0.99±0.09
2	83	5.12×10^{-5}	1.08±0.19
2.5	84	4.67×10^{-5}	1.00±0.30
3	77	7.91×10^{-5}	1.67±0.01

The results indicate that although levels of cell kill observed in this experiment are fairly small, only at the highest MNNG concentration was a significant rise in tf observed. At the same concentration, MNNG causes ~ 5 fold increases in Mud1(Ap^r, *lac*) tf (chapter 4). In contrast, the *his* reversion frequency, which was 5.02×10^{-8} in the control subculture, rose to such a high extent in all of the MNNG treated subcultures as to make colony counts impossible (> 2000 colonies/plate) indicating that these levels of MNNG, even for a relatively short exposure time, are highly mutagenic.

The slow growth of *lac*$^+$ colonies observed in TA1975 Mud1(Ap^r, *lac*) was not observed with TA1950 Mud1(Ap^r, *lac*) indicating that the slow growth rate was indeed related to the *rfa* mutation and may involve the inefficient attachment of lactose permease to the cell membrane of *rfa* mutants, or a reduced activity of the protein in the altered membranes. Due

to lack of time TA1535 Mud1(Ap^r, *lac*) was not studied in transposition assays.

These results, although preliminary, seem to suggest that although Mud1(Ap^r, *lac*) does transpose in *Salmonella* at frequencies comparable to those observed in *E. coli*, and shows the same temperature sensitivity as in *E. coli*, the transposogenic effect of MNNG is less than observed in *E. coli*. Mutations rendering the cells more sensitive to the mutagenic effects of carcinogens do not increase the sensitivity towards a transposogen, neither does the induction of mutagenesis correspond with an induction of transposition, the frequency of *his* reversion being raised significantly at doses of MNNG which have no effect on tf. This is strong evidence against the mutagenicity of MNNG being responsible for transposogenesis.

CHAPTER 6: INVESTIGATION OF A SHORT-TERM TEST FOR MUTAGENIC AND NON-MUTAGENIC CARCINOGENS, BASED ON THE INDUCTION OF INTRA-CHROMOSOMAL RECOMBINATION IN YEAST.

6.1. Introduction.

As it became obvious that the long term aims of this project, i.e. the development of a simple, short-term test, capable of identifying potential environmental carcinogens not detected by conventional short-term tests, were unlikely to be fulfilled using a system based on the measurement of transposition frequencies, it was decided to dedicate the final months of the project to the investigation of a newly developed system, recently reported to be able to identify, and distinguish between both conventional mutagens and non-mutagenic carcinogens (Schiestl, 1989).

On a similar basis to the development of transposition assays, the new system was designed with the hope of identifying potential carcinogens capable of causing large scale genome rearrangements, in this case the deletion of a recombinant plasmid, integrated into the *HIS3* gene of *Saccharomyces cerevisiae*, via an intrachromosomal recombination event.

The plasmid pRS6 (Schiestl, 1989) is a derivative of pBR322 which contains an internal fragment of the *S. cerevisiae HIS3* gene, along with a copy of the *LEU2* gene. The plasmid is cut within the *HIS3* fragment and integrated into the genome of the recipient yeast strain at the *HIS3* locus, resulting in a duplication of the *HIS3* gene, in which one allele has a deleted 5' end. The copies share about 400bp of homology and are separated by an active *LEU2* gene and the pBR322 sequences (figure 6.1). The recipient strain used in this work is a diploid strain of *Saccharomyces cerevisiae* which has a deletion of one copy of the *HIS3* gene (*his*3-Δ200) and mutations rendering both copies of the *LEU2* gene inactive (*leu*2-3, 112/*leu*2-Δ98). On integration of the plasmid pRS6, the strain becomes phenotypically his$^-$, leu$^+$ and the integrated plasmid may be selected for by growth on a minimal medium

lacking leucine.

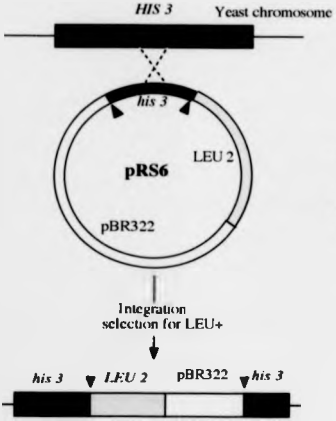


Figure 6.1. Integration of pRS6 into *S. cerevisiae* chromosome at the *HIS3* locus, generating a *his3* duplication flanking the pBR322 and *LEU2* sequences.

Reversion to a *HIS3* genotype may occur by three potential mechanisms (figures 6.2, 6.3). The first such mechanism is via a simple plasmid excision event, resulting in a complete *HIS3* gene and an intact, extrachromosomal copy of pRS6. However, in experiments using an integrated plasmid with a yeast origin of replication, although intact plasmids could be recovered from *HIS3*+ revertants, they occurred at a frequency 100 fold lower than the reversion frequency, ruling out plasmid excision as the major reversion pathway (Schiestl, Igarashi and Hastings, 1988).

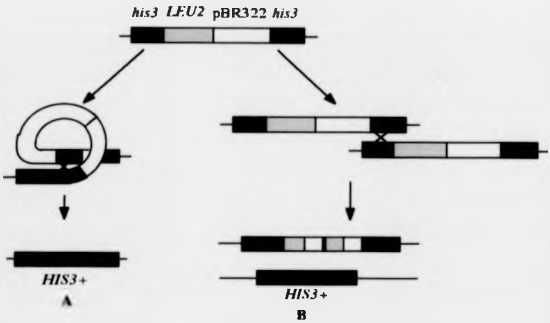


Figure 6.2. Possible mechanisms of DEL recombination; **A**, plasmid excision via homologous recombination between the *his3* alleles and **B**, chromatid exchange between alleles on sister chromatids.

The second possible mechanism is an unequal sister chromatid exchange event, resulting in one intact copy of the *HIS3* gene and one allele containing two copies of the pRS6 insert. When chromosomal DNA from *HIS3+* revertants was probed with pBR322 DNA in Southern blotting experiments, the characteristic double band on agarose gels, expected from a double insert, was not observed in any of the 25 revertants investigated (*ibid*). Thus it was proposed that the major mechanism of *HIS3+* reversion was via an intrachromosomal recombination event, possibly initiated by a double stranded break, extended to a gap by exonuclease and subsequently repaired by gene conversion from the sister chromatid as a donor (figure 6.3). Segregation of the chromatids now results in one *HIS3* revertant showing a

HIS+, leu- phenotype, which is in fact observed in 99% of all *HIS*+ revertants.

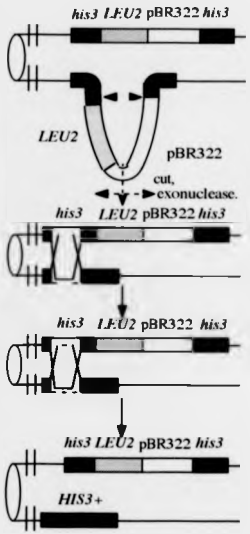


Figure 6.3. Deletion of the integrated plasmid pRS6 by sister chromatid conversion, resulting, after segregation, in a revertant with a HIS+, leu- phenotype. It is proposed that a double strand break initiates the recombination event which is extended by an exonuclease to a gap. The gap is repaired by gene conversion from the sister chromatid as donor (Schiestl, 1989).

In addition to the system for measuring intrachromosomal recombination ('deletion' or 'DEL'), the diploid strain employed in this work, RS112, has two further mutations, in the two copies of the *ADE2* gene (*ade2*-401, *ade2*-101), resulting in an ade- phenotype. These allow the strain to be employed in the detection of chemicals capable of inducing *inter*chromosomal

recombination (ICR) between the *ade2* heteroalleles. This type of assay has been used to detect a wide range of carcinogens and mutagens, including alkylating agents, aromatic amines and radical forming species (for a review see Zimmerman *et al*, 1984). Several carcinogens not detected by the Ames *Salmonella* test have given positive results, notably auramine, thioacetamide and safrole (the last compound is weakly positive) (Simmon, 1979).

This type of mitotic recombination occurs in two ways. The first is a reciprocal recombination between non-sister chromatids, with the formation of one double mutant allele, and one wild type gene. In most cases however, recombination occurs via mitotic gene conversion between two alleles where a non-reciprocal transfer of small segments of genetic information (between several hundred and a thousand base pairs) occurs (Hurst and Fogel, 1964; Zimmerman, 1968).

The genetic endpoint of the ICR test (a reversion to adenine prototrophy) is the result of a complex set of induced repair pathways (Zimmerman *et al*, 1984), and as such does not show any marked specificity to the type of mutagen used as is observed in the Ames test. However, the development of repair deficient strains in order to increase sensitivity has, as a result, proved impractical (*ibid*).

Initial results by Schiestl, using the DEL recombination system, have indicated that not only can recognised genotoxic chemicals be detected with high efficiency, but that chemicals which are known or suspected carcinogens, yet which have shown no genotoxic activity in other short term tests, may cause increases in the frequency of DEL recombination (Schiestl *et al*, 1989). The results of the initial experiments are summarised in table 6.1.

Table 6.1. Response of three short-term tests to a variety of carcinogenic agents.

Carcinogen	DEL	ICR	Ames
UV	+	+	+
Methyl methane sulphonate	+	+	+
Ethyl methane sulphonate	+	+	+
4-Nitroquinoline-N-oxide	+	+	+
Nitrogen mustard	+	+	+
Aflatoxin B1	+	+	+
Formaldehyde	+	(+)	-
Safrole	+	-	-
Ethionine	+	-	-
Urethane	+	-	-
Auramine	+	-	-
Carbon tetrachloride	+	-	-
Carbon dichloride	+	-	-
Cadmium chloride	+	-	-
Cadmium sulphate	+	-	-
Aniline	+	(+)	-

3-Amino triazole	+	+	-
Acetamide	+	+	-
Thioacetamide	+	+	-
Thiourea	+	+	-
DDE	+	+	-
Ethylene thiourea	+	+	-

+ positive; (+) weakly positive; - negative.

Although based on a limited number of chemicals the results obtained show clearly that a positive response in the DEL recombination assay is obtained for a range of carcinogens which would be classified as non-genotoxic, and thus appear as false negatives, if tested with the standard set of Ames tester strains currently recommended (Maron and Ames, 1983). Additionally, two mutagenic chemicals, hydroxylamine and sodium azide, which show no significant carcinogenic activity, gave positive results in the ICR test with no induction of DEL. A third mutagen which is non carcinogenic, bromouracil, gave a negative result in both tests (Schiestl *et al*, 1989).

Taken as a whole, the results indicate a sensitivity of 0.96, with only one false negative out of 27 carcinogens tested, and a specificity of 0.8, with one false positive out of 5 non carcinogens. The overall accuracy, as defined by De Serres and Ashby (1981), was 0.93.

The sensitivity of the Ames test, based on a review of updated databases from long term tests, has recently been estimated at as low as 0.45 (Tennant *et al*, 1987). Given that many of the chemicals so far tested on the DEL system were chosen because of their lack of

response in the Ames test, there is much to recommend a full investigation into the usefulness of the DEL system as a predictive test for carcinogenicity.

An intriguing feature of the DEL system is the different dose response profile for the test obtained with carcinogens not detectable in the Ames test, from that obtained with conventional mutagenic carcinogens. The Ames positive carcinogens induce both DEL and ICR equally well in a linear, dose dependant manner over a wide range of concentrations. The Ames negative carcinogens however, preferentially induce DEL recombination, and show a threshold concentration of carcinogen below which no induction of DEL is observed. The maximum level of induction of DEL by the Ames negative chemicals is much less than that obtained with the Ames positive agents (~10x compared to ~100-1000x) (Schiestl, 1989).

The enzymology of mitotic recombination in the lower fungi is poorly understood. The best characterised enzymes are the *REC1* gene product of *Ustilago maydis*, an ATPase which catalyses homologous strand transfer in a similar fashion to the recA protein of *E. coli* (Kmiec and Holloman, 1982) and the *CDC9* gene product of *S. cerevisiae* which is a DNA ligase essential for the completion of induced recombination (Fabre and Roman, 1979). Additionally, the *RAD3* gene product of *S. cerevisiae* has been purified and shown to possess DNA-dependent ATPase and helicase activities (Sung *et al*, 1987b).

A large number of mutants which affect the frequency of recombination have been described (Kunz and Haynes, 1981; Orr-Weaver and Szostak, 1985). These fall into five categories; those selected

for their ability to influence genetic exchange, which may affect recombination frequencies (*con, cor, MIC, rec*) and four classes with plieotropic effects which have been selected on the basis of (a) chemical or radiation sensitivity (*rad, mms*), (b) altered mutability (*mut, rem*), (c) defective sporulation (*spo, mei*) and (d) cell cycle deficiencies (*cdc*).

Mitotic recombination in fungi appears to be a consequence (mechanism?) of DNA repair. Many pathways for the repair of mismatches, errors, double strand breaks, pyrimidine dimers and other lesions in DNA are known to result in recombination. Many of the mutations which affect recombination are in genes which are known to encode repair functions. As previously mentioned, the *CDC9* gene product of *S. cerevisiae* is a DNA ligase. The level of its gene transcript reaches a maximum at the G1/S phase boundary of the cell cycle, just before the onset of DNA synthesis (White *et al*, 1986). *cdc*9 Mutants are defective in joining Okazaki fragments and are thus sensitive to DNA damaging agents. In a study using a temperature sensitive *cdc*9 mutant, Norrander *et al* (1986) found that in the absence of the *CDC9* gene product, DNA replication (in this case of the yeast 2μm plasmid) was complete apart from the ligation of single-strand interruptions (mostly breaks in a single phosphate-ester bond rather than gaps due to missing nucleotides). Mutations in *CDC9* are lethal due to the arrest of the cell cycle during the G2 phase, presumably to allow for the repair of damaged DNA before progression into mitosis. The *RAD9* gene product is required for this arrest (Schiestl *et al*, 1986).

*cdc*9 Mutants, in experiments performed using conditionally lethal temperature sensitive mutants, show a hyper-rec

phenotype with elevated basal levels of mitotic inter and intrachromosomal recombination (Kunz and Haynes, 1981; Schiestl and Prakash, 1988), although levels of *induced* mitotic recombination may be reduced (Kunz and Haynes, 1981). Schiestl and Prakash (1988) reported that temperature sensitive *cdc*9 mutants containing the *his3*Δ3', *his3*Δ5' duplication (for measuring DEL frequencies), showed approximately 30 fold increases in basal levels of intrachromosomal recombination even when grown at the permissive temperature. This increased frequency of DEL recombination is dependant on the *RAD1* gene product which is required for the incision of UV-damaged DNA. A difference in the mechanisms of inter and intrachromosomal recombination is evident, as *rad1* mutants of *S. cerevisiae* show decreased levels of intrachromosomal (DEL) recombination (*ibid*) while levels of ICR are unaffected (*ibid*, Dicaprio and Hastings, 1976).

Mutations in the *RAD6* group are defective in error prone repair. *rad6* mutants are unable to repair double and single strand breaks induced by chemical mutagens and diploids homozygous for *rad6* show increased levels of UV, gamma ray and methyl methane sulphonate (MMS) induced recombination (Chlebowicz and Jachymczyc, 1979; Kunz and Haynes, 1981).

Mutants of *RAD18* accumulate single and double strand breaks after x-ray irradiation and show an increased level of post UV-irradiation single strand breaks over RAD wild-type strains. Diploids homozygous for *rad18* show elevated levels of induced recombination.

Mutations in the *RAD52* group of genes (*rad52*, *rad54*, *rad57* and *rad51*) also cause defects in the repair of double strand

breaks, however genes in this group are involved in *recombinational* repair and mutants have decreased levels of mitotic recombination (*ibid*; Schiestl and Prakash, 1988; Orr-weaver and Szostak, 1985).

Intrachromosomal recombination depressed by the *rad1* mutation is further depressed synergistically in a *rad1, rad52* double mutant, indicating that the two genes act in two different recombinational pathways (Schiestl and Prakash, 1988).

The hyper-rec phenotype of *cdc9* mutants, and other mutants with defective strand break repair is especially interesting when compared to the karyotype of cells from patients suffering from Bloom's syndrome (BS). BS cells have a defective DNA ligase, and show elevated levels of sister chromatid exchanges and other chromosomal abberations (see chapter 1.1.3). It would appear that nicks and breaks in DNA, of the type unrepaired in cells with defective ligase enzymes, are highly recombinogenic. In mammals, genetic rearrangements of this type, by transferring proto-oncogenes to regions where the structure or expression is altered, are thought to be important steps in carcinogenesis (see chapter 1 for a detailed discussion). Patients with BS suffer an approximate 100 fold increased incidence of all types of cancer (Cairns, 1981).

As discussed previously (chapter 1.1.3), high levels of intracellular superoxide radical can induce nicks, double strand breaks and base liberations in cellular DNA. Superoxide radical induced cellular damage is implicated in a number of disease states, including carcinogenesis, and thus any new test for carcinogens must be able to detect oxidative mutagens as such. The strain RS112 had not previously

been tested with this class of mutagens, and thus it was decided to investigate the responses of the two types of recombination assay in RS112 to a selected group of oxidative mutagens, some of which had been tested with transposition tester strains previously in this work.

6.2. Results.

6.2.1. Reisolation and Characterisation of RS112.

In order to revive the RS112 strain, received as a slant on YPAD agar in a sealed bottle, cells from the slant surface were aseptically streaked, using a sterile loop, onto a fresh YPAD agar plate and incubated at 30°C overnight.

10 isolated colonies from this plate were picked and streaked onto separate SC, SC-his and SC-ade plates, to check for a his , ade phenotype. After incubation at 30°C for 24 hours, each of the 10 isolates showed good growth on SC plates, with only a small number of colonies due to spontaneous reversion appearing on the SC-his and SC-ade plates.

Isolated colonies from the SC plates of the three isolates which showed the lowest apparent levels of *HIS+* and *ADE+* reversion, isolate nos 1, 4 and 6, were picked and used to seed 3 separate flasks containing 20ml each of sterile SC-leu medium.

After overnight growth at 30°C, agitated at 200 rpm, cells from the three cultures were pelleted and resuspended in sterile distilled water containing 1mM EGTA (EGTA was added in order to chelate Ca^{2+} ions in the medium, thus inhibiting "clumping" of yeast cells). The

resuspended cultures were vortexed vigorously for 1 minute to aid dissociation of clumps, and diluted in 4x10 fold steps, in sterile distilled water, to a dilution factor of 10^4.

In order to measure the total number of viable cells per ml of culture for each of the three isolates, 100μl aliquots of the 10^4 fold dilutions were added to 2.6 ml of sloppy top agar (kept molten at 47°C), rapidly mixed, poured onto an SC agar plate and spread evenly across the surface by gently tilting the plate. Duplicates were performed for each isolate. Additionally a third 100μl aliquot of each 10^4 dilution was spread directly onto the surface of an SC plate, using a sterile glass rod, as a control to check for any loss of viability when using sloppy top agar.

The frequencies of intrachromosomal (DEL) and interchromosomal (ICR) recombination for each strain were also estimated by plating of the appropriate dilutions (10^1 fold for DEL and 10^0 fold for ICR) in sloppy top agar, onto SC-his and SC-ade agar plates. The numbers of *HIS+* and *ADE+* revertant colonies growing gave direct estimates of DEL and ICR respectively. Once again duplicates were performed, with a third control for loss of viability with sloppy top agar.

The number of colonies on each plate was counted after 72 hours incubation at 30°C. In each case the apparent colony count for the plates spread using sloppy top agar was higher than that on the 'control' plate, due to a much better dispersion of the colonies in top agar. Future platings were performed using the sloppy top agar method uniquely.

The results are given in table 6.2. Only counts from plates

spread using sloppy top agar have been used. Numbers in brackets represent the total number of colonies counted in each case.

The frequencies of spontaneous *HIS+* and *ADE+* reversion (ie DEL and ICR recombination), are entirely consistent with those reported by Schiestl (Schiestl, 1989). Isolate No1, with the lowest levels overall of spontaneous recombination, was used in all future experiments.

Table 6.2. Cell counts and spontaneous reversion frequencies of overnight cultures of 3 RS112 isolates.

Isolate No	Total viable cells/ml culture	*HIS+* revertants per 10^8 cells	*ADE+* revertants per 10^8 cells
1	5.2 x 10^7 (1040)	1.49 (155)	1.99 (207)
4	4.0 x 10^7 (800)	1.41 (113)	4.29 (343)
6	3.95 x 10^7 (790)	2.53 (200)	2.51 (198)

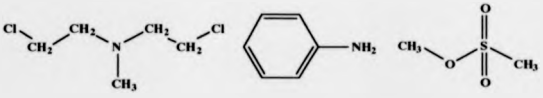
In order to check for the HIS+, leu- phenotype expected from a *HIS+* revertant arising via the DEL recombination model proposed by Schiestl (*ibid*), 300 of the *HIS+* colonies obtained in the previous experiment were picked onto sectored SC-his and SC-leu agar plates.

The plates were inspected after 48hrs incubation at 30°C. Of 228 colonies which grew on SC-his plates, only 4 also grew on SC-leu plates. Thus 98.2% of *HIS+* revertants showed a HIS+, leu- phenotype. This is again consistent with the 99% HIS+, leu- phenotype reported for *HIS+* revertants of RS112 (*ibid*), and supports the hypothesis that *HIS+* reversion of RS112 occurs mainly by an intrachromasomal recombination

of the two *his*3 alleles, via sister chromatid conversion (Schiestl *et al*, 1988).

6.2.2. Confirmation of Responsiveness of RS112 to Ames Positive and Ames Negative Carcinogens.

In order to ensure that results obtained by Schiestl and colleagues in the United States were reproducible in our laboratories; four chemicals were chosen which were reported to give a positive response in DEL and/or ICR recombination assays using RS112 (Schiestl, 1989; Schiestl *et al*, 1989).



A B C

Figure 6.4. Chemical structures of A Nitrogen Mustard, B Aniline, and C Methyl methane sulphonate.

Two of the chemicals, methyl methane sulphonate (MMS) and nitrogen mustard (NM) (figure 6.4), are strongly mutagenic in the Ames test (McCann *et al*, 1975). MMS and NM are both powerful alkylating agents, which cause alkylation of proteins and nucleic acids, and induce cancers in a variety of organs after intravenous injection into mice (Lawely, 1976).

The second two chemicals, aniline (figure 6.4) and cadmium chloride ($CdCl_2$), are both negative in Ames mutagenicity assays using all five of the currently recommended strains (McCann *et al*, 1975). However both chemicals induce cancer in multiple organ sites in rats (Ashby and Tennant,

1988; Sittig, 1985), and a 2.5 fold increased risk of renal cancer has been reported for humans occupationally exposed to Cadmium (Sittig, 1985). Both chemicals are reported to give a positive response in DEL recombination assays using RS112, with little or no induction of ICR (Schiestl *et al*, 1989).

For each of the experiments, an isolated colony of RS112, from a YPAD agar plate seeded and incubated at 30°C for no more that 48 hours, was selected and divided into two. Half of the colony was used to seed the culture flask containing 20ml of SC-leu medium, which was subsequently grown at 30°C, agitated at 200rpm, overnight. The second half of the colony was streaked onto SC-his and SC-ade agar plates to check for a his⁻ ade⁻ phenotype.

After overnight growth, cells from the culture flask were counted using a haemacytometer, under a light microscope, at 1000x magnification. The culture was diluted to a concentration of $2x10^6$ cells/ml with fresh SC-leu medium, and added in 10ml aliquots to 25ml universal sterile disposable bottles.

Various concentrations of the chemical to be tested were added to these subcultures, which were then incubated at 30°C/300rpm for 18 hours. After treatment, cells from each subculture were pelleted, washed once with either 5% sterile sodium thiosulphate (alkylating agents) or sterile distilled water, a second time with sterile distilled water, resuspended in 1mM EGTA, vortexed and counted using a haemacytometer, to give an estimate of the chemical's effect on growth rate of the subculture (in terms of the number of generations of cells in the subculture). Cells were then pelleted a fourth time, and resuspended in 1ml of sterile distilled water.

Each subculture was then diluted in 5x 10 fold steps, to a final

dilution of 10^{-4} of the original subculture. Dilutions were plated as previously, in triplicate, to give measures of (i) % variable cells; (ii) no. *HIS*+ revertants; (iii) no. *ADE*+ revertants for each subculture.

Concentrations of each of the four chemicals initially tested were chosen by direct reference to those used by Schiestl *et al* (1989). Four concentrations were used for NM (0, 0.1, 0.5, 1μg/ml) and MMS (0, 50, 150 and 250μg/ml subculture). Six concentrations were used for aniline (0, 1, 2, 3, 4, 5mg/ml) and $CdCl_3$ (0, 50, 100, 150, 200, 300μg/ml). Results of these experiments are shown in tables 6.3 to 6.6, and graphically in figures 6.5 to 6.8.

Tables show the number of generations achieved by each subculture, the percentage of viable cells in each case, and the *HIS*+ and *ADE*+ reversion frequencies, along with their relative values when compared to the untreated subculture. Numbers in brackets represent the number of colonies counted for each calculation. Relative reversion frequencies (rrf's) are quoted ± the standard deviation between triplicate *HIS*+ or *ADE*+ plate counts (N.B. This is not a measure of the experimental reproducibility, only an indication of *intra*-experimental errors).

Table 6.3. Effect of NM on *HIS*+ and *ADE*+ reversion frequencies of RS112.

NM in μg/ml	Generations	Survivors in %	*HIS*+/10^4 cells	rrf±SD (*HIS*+)	*ADE*+/10^8 cells	rrf±SD (*ADE*+)
0	4.35	100(756)	1.81(1368)	1.00±0.02	3.57(270)	1.00±0.07
0.1	4.41	84(655)	18.9(789)	10.45±0.22	15.7(1022)	4.40±0.10
0.5	3.90	48(327)	58.5(1606)	32.30±0.76	48.1(1330)	13.47±0.15
1.0	3.37	54(231)	74.7(1530)	41.25±2.98	53.9(1106)	15.12±0.31

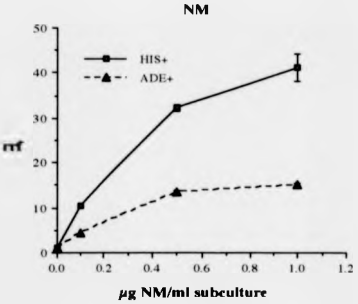


Figure 6.5. The effect of Nitrogen mustard (NM) on *HIS*+ and *ADE*+ reversion of RS112. Error bars indicate the standard deviation between colony counts on triplicate plates.

Table 6.4. Effect of MMS on *HIS*+ and *ADE*+ reversion frequencies of RS112.

MMS in μg/ml	Generations	Survivors in %	*HIS*+ /10⁴ cells	rrf±SD (*HIS*+)	*ADE*+ /10⁴ cells	rrf±SD (*ADE*+)
0	4.64	100(750)	1.31(1474)	1.00±0.03	1.27(143)	1.00±0.23
50	4.41	33(415)	11.4(4735)	8.73±0.17	24.2(1063)	19.02±2.32
150	3.83	3(30)	52.9(1593)	40.41±1.04	51.6(251)	40.65±4.33
250	2.83	1(6)	-	-	-	-

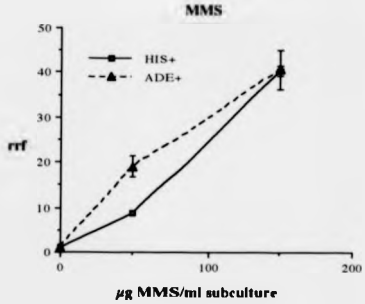


Figure 6.6. The effect of Methyl methane sulphonate (MMS) on *HIS*+ and *ADE*+ reversion of RS112. Error bars indicate the standard deviation between colony counts on triplicate plates.

Table 6.5. Effect of Aniline on *HIS*+ and *ADE*+ reversion frequencies of RS112.

Aniline in mg/ml	Generations	Survivors in %	*HIS*+ /10⁸ cells	rrf±SD (*HIS*+)	*ADE*+ /10⁵ cells	rrf±SD (*ADE*+)
0	4.36	100(281)	1.23(702)	1.00±0.05	1.52(87)	1.00±0.15
1	4.31	100(790)	0.96(659)	0.78±0.06	2.01(140)	1.32±0.23
2	3.42	52(1539)	1.40(242)	1.14±0.09	1.20(184)	0.79±0.13
3	1.57	43(354)	1.26(449)	1.03±0.12	0.62(22)	0.41±0.21
4	0	34(222)	1.96(246)	1.59±0.11	0.85(16)	0.56±0.34
5	0	29(1296)	7.22(872)	5.87±0.21	1.31(15)	0.86±0.23

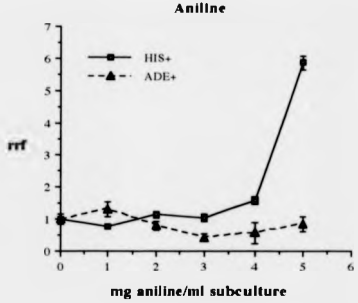


Figure 6.7. The effect of aniline on *HIS*+ and *ADE*+ reversion of RS112.

Table 6.6. Effect of $CdCl_2$ on *HIS*+ reversion frequency of RS112.

$CdCl_2$ in µg/ml	Generations	Survivors in %	*HIS*+ /10^4 cells	rrf±SD (*HIS*+)
0	5.26	100(985)	2.54(2163)	1.00±0.01
50	0.53	60(233)	2.31(598)	0.91±0.03
100	0.13	33(90)	3.37(375)	1.47±0.02
150	0.33	31(810)	4.48(313)	1.76±0.02
200	0.23	14(545)	5.58(213)	2.20±0.29
300	0	0		

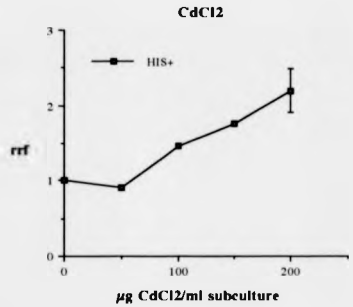


Figure 6.8. The effect of $CdCl_2$ on *HIS*+ and *ADE*+ reversion of RS112.

Despite the small number of points obtained for the MMS data, due partly to a high level of toxicity at the highest dose, and the apparent plateau of response at the highest dose of NM; it is clear that both of the mutagenic, Ames positive carcinogens gave large positive responses in both DEL and ICR recombination assays at all doses tested. Although the number of doses is too small to be certain, there would appear to be no threshold of dose below which no response is observed. In contrast, the data for the non-mutagenic, Ames negative carcinogen aniline, clearly shows a dose threshold, of about 4mg aniline per ml subculture, below which no response in either DEL or ICR was observed. Above this dose level a large increase in DEL was observed although there was no increase in ICR. This threshold corresponds to the dose where a high toxicity effect is also observed, i.e. a large inhibition of growth and also a high level of cell kill.

The data for the $CdCl_2$ experiment is less clear cut, no data were obtained for ICR due to the high toxicity of the chemical at all doses (due to the lower frequency of ICR the number of surviving cells is much more critical to the gathering of reliable data than for DEL, which generally occurs at a 10 fold higher frequency). The data obtained for DEL however, do appear to show a threshold dose of around 50μg per ml of subculture, below which no response was seen and above which an increase in the frequency of DEL was observed. The maximum induction observed was slightly lower than that reported by Schiestl *et al* (*ibid*), possibly due to the higher level of toxicity observed in our experiments.

Generally, the data obtained from these four experiments indicated that the data obtained by Schiestl *et al* in the U.S. were reproducible in our laboratories.

It was thus decided to extend our investigations of the new test to the small group of chemicals, all oxidative mutagens, in which we were interested.

N.B. Due to the corroborative nature of the above results, the experiments were not repeated. However, all subsequent results obtained with strain RS112 are representative of at least two separate experimental repetitions unless otherwise indicated. thus any results reported are reproducible.

6.2.3. Investigations into the Effects of a Group of Oxidative Mutagens on DEL and ICR in RS112.

The first oxidative chemical which we wished to test on the RS112 DEL/ICR system was the herbicide methyl viologen (paraquat, PQ) (figure 6.9).

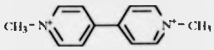


Figure 6.9. Chemical structure of Paraquat.

In *E. coli*, PQ undergoes a redox cycle whereby it is reduced by an NADPH dependent diaphorase enzyme and subsequently reoxidised *in situ* from molecular oxygen (figures 6.10, 6.11). Greater than 95% of the O_2^- superoxide radical thus generated is intracellular, although a small amount of reduced paraquat (PQ^+) can effuse and generate O_2^- outside the cell (Hassan and Fridovich, 1979). PQ is therefore a convenient tool for specifically raising intracellular levels of O_2^-.

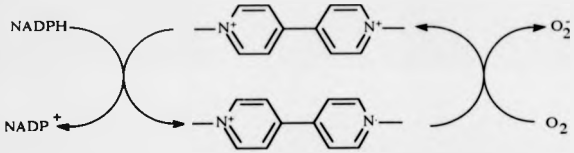


Figure 6.10. The one electron reduction of a paraquat to the paraquat radical by an NADPH dependent diaphorase enzyme, and and its subsequent autoxidation with dioxygen generating a superoxide radical.

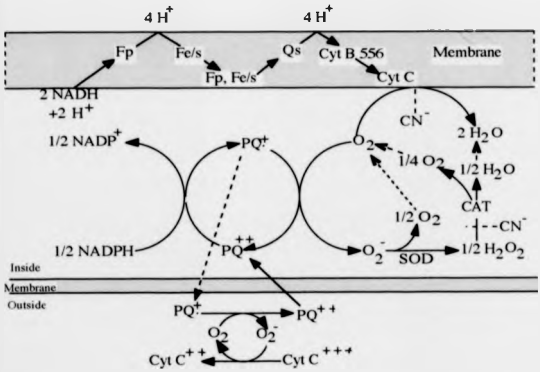


Figure 6.11. Schematic representation of the pathways of dioxygen reduction in the presence of paraquat. *Cyt C*, cytochrome C; *CAT*, catalase; *SOD*, superoxide dismutase. Adapted from Farrington *et al.* (1973).

Treatment with PQ has been shown to induce *de novo* protein synthesis of the manganese containing superoxide dismutase enzyme (SOD) in both *E. coli* and *S. typhimurium*, at the level of transcription (Hassan, 1982). PQ has also been reported to be mutagenic to the Ames tester strains TA98 and TA100 (*ibid*), although detection of PQ as a mutagen, using the

standard Ames test methods, is hampered by the high toxicity of the chemical and the results obtained using TA100 and TA98 have been questioned.

Results, even employing the strain TA102 specifically designed for the detection of oxidative mutagens, are often inconclusive (Levin *et al*, 1984). Paraquat has also been reported to induce mitotic gene conversion (ICR) in *S. cerevisiae* diploid strains hetroallelic at the *ADE2* and *HIS1* loci, at levels which allowed a high percentage of cell survival (Parry, 1973).

PQ was detected as weakly transpogenic in previous experiments using the transposition tester stains MC4100 Mud1(Ap^r, *lac*) and UWE103(pBR322) (Chapter 3.3).

Taking into account the high toxicity of PQ to prokaryotes, (100% kill of MC4100 Mud1(Ap^r, *lac*) was observed at a PQ concentration of 1mM), initial experiments using RS112 were performed with a range of PQ concentrations 0.1 to 250μg PQ/ml subculture (250μg/ml $\approx$ 1mM).

After incubation of the subcultures at 30°c for 17hrs it was evident from cell counts that even at the highest PQ concentration there was no inhibition of growth. Cells from the two highest PQ concentrations (150 and 250μg/ml) and the minus PQ control, were plated out as previously, to determine if any induction of DEL or ICR had occurred. Colony counts from these plates showed no induction of either DEL or ICR at the highest dose (rrf *HIS*+ 1.08±0.02, rrf *ADE*+ 0.98±0.04), and no kill effect.

A second experiment was performed in which the PQ concentration was raised up to 1.5mg/ml. Once again very little growth inhibition was observed at even the highest PQ concentration. However colony counts from plating of the control and highest two PQ concentrations

yielded the following data:

Table 6.7. Effect of low level paraquat on *HIS*+ and *ADE*+ reversion of RS112.

PQ in mg/ml	Generations	Survivors in %	*HIS*+/10⁴ cells	rrf±SD (*HIS*+)	*ADE*+/10⁸ cells	rrf±SD (*ADE*+)
0	4.83	100(992)	1.53(1519)	1.00±0.04	3.39(336)	1.00±0.04
1.0	4.45	99(765)	1.86(1171)	1.21±0.03	3.39(259)	1.00±0.01
1.5	4.37	75(487)	2.07(1125)	1.35±0.02	3.26(186)	0.96±0.03

The results show a small increase in *HIS*+ reversion at both concentrations of PQ, and some cell kill effect at the highest level. No increase in *ADE*+ reversion was observed.

The level of DEL induction was too low for any firm conclusion to be drawn. A further experiment was undertaken with subcultures of RS112 with a range of PQ concentrations up to 35mg/ml (equivalent to 137mM).

The results obtained are given in table 6.8 and shown graphically in figure 6.12.

The results show a linear increase in *HIS*+ reversion with increasing PQ concentration. No increase in *ADE*+ reversion was observed over the concentration range used. This is in contrast to the findings of Parry (*ibid*) who reported a 60 fold increase in the level of *ADE2*+ reversion of *S. cerevisiae* at a PQ concentration of 0.6mg/ml.

Table 6.8. The effect of high level PQ on RS112.

PQ in mg/ml	Generations	Survivors in %	*HIS*+/10⁴ cells	rrf±SD (*HIS*+)	*ADE*+/10⁵ cells	rrf±SD (*ADE*+)
0	4.64	100(1465)	1.00(488)	1.00±0.21	2.50(366)	1.00±0.21
5	3.35	80(612)	1.49(304)	1.49±0.11	2.45(150)	0.98±0.02
15	2.98	52(473)	2.47(389)	2.47±0.27	2.60(123)	1.04±0.11
30	2.13	37(263)	4.97(435)	4.97±0.31	2.23(585)	0.89±0.00
35	1.98	24(237)	5.48(432)	5.48±0.40	2.03(480)	0.81±0.13

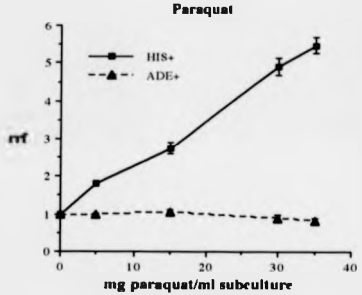


Figure 6.12. The effect of Paraquat on *HIS*+ and *ADE*+ reversion in RS112

The experimental procedure employed in Parry's study was slightly different to that used for RS112, in that cells were incubated in the presence of paraquat as a suspensionin sterile saline solution. Cells were thus

unable to undergo replication and growth during the incubation period, and incapable of synthesizing the protective SOD enzymes normally produced in response to oxidative shock (presumably the supply of electrons (NADPH) required for the production of O_2^- from PQ was supplied from sufficient cellular pools). These differences may explain the difference in toxicity of PQ in the two studies (different basal levels of SOD activity in the two *S. cerevisiae* strains may also have some effect), however at the levels of cell kill at which *ADE+* reversion was induced in Parry's study (~10-90% kill), no induction of *ADE+* reversion was observed in our study. The induction of *ADE+* reversion in heteroallelic yeast strain by PQ may thus be restricted to late log phase or stationary cultures as used by Parry. Any recombinogenic DNA intermediates induced by PQ treatment may be efficiently repaired by growing cells.

The ability of the DEL system to identify PQ as mutagenic, although at high concentrations, was extremely encouraging. Rather than investigating the effect of PQ further, it was decided to carry on and test other oxidative mutagens.

The next chemical investigated was mitomycin C (MC) (figure 6.13). MC is a naturally occurring anticancer agent with a high toxicity towards hypoxic tumour cells relative to their oxygenated counterparts (Pristos and Sartorelli, 1986). MC is activated *in vivo* to a bifunctional alkylating agent with two nucleophilic carbonium centres (figure 6.13), and subsequently acts as a crosslinking agent. The toxicity of MC is thought to be directly related to the formation of crosslinks, as structurally related compounds which react with DNA without forming crosslinks are much less cytotoxic (Green, 1979). The preferential toxicity of MC towards hypoxic cells is thought to be due to a partial reduction, under aerobic conditions, to the semiquinone species, and a subsequent oxygen cycling whereby the semiquinone is reoxidised by molecular oxygen, thus forming superoxide

radicals in a fashion analogous to Paraquat (Pristos and Sartorelli, 1986) (figures 6.10, 6.11)

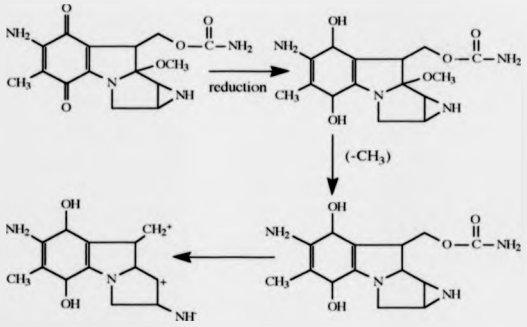


Figure 6.13. The chemical structure of Mitomycin C and its *in vivo* activation to give two reactive carbonium centres.

Mitomycin C is detected as weakly mutagenic in a number of short term tests, however the mutagenicity of MC is dependent upon an intact excision repair and recombinational repair pathway (Green, 1979). A detailed study on the mechanism of repair of crosslinks was made by Cole (1973) who studied the repair of trimethyl psoralen crosslinks in *E.coli*. A repair mechanism involving the coordinated action of incision and recombination repair pathways was proposed (figure 6.14).

If the mutagenicity of MC is indeed due to the action of an error-prone repair mechanism involving recombination, then the yeast strain RS112 would be expected to respond to MC treatment with an increase in the frequencies of both DEL and ICR. MC has been reported to induce mitotic recombination in *S. cerevisiae* (Holliday, 1964), it is also detected as

mutagenic by the Ames *Salmonella* tester strain TA102 which was developed with the specific aim of identifying oxidative mutagens (Levin *et al*, 1984), although TA102 does have the plasmid pKM101 which confers error prone repair to the cell (McCann *et al*, 1975). MC also causes chromosome abberations, including sister chromatid exchanges, in cultured human lymphocytes (Garner and Martin, 1979).

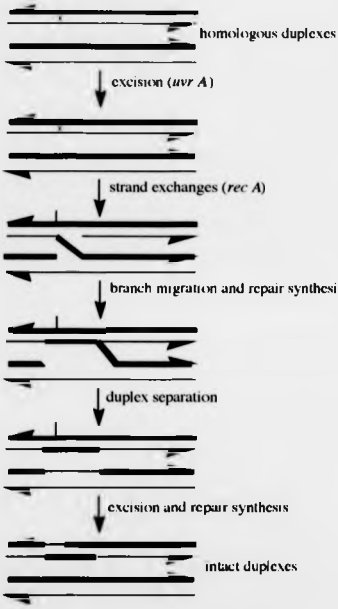


Figure 6.14.

Possible mechanism for the repair of mitomycin C-DNA crosslinks (Cole, 1973). Two incisions are made near each crosslink (*uvrA* controlled). A nuclease (possibly DNA polymerase I) widens the gap exposing single stranded DNA. The following strand exchanges (*recA* controlled) between homologous duplexes insert a base sequence complimentary to the strand still carrying the partially excised crosslinking residue, which is fully excised after restoration of the twin helical DNA structure.

In transposition assays using MC, it was highly toxic towards *E.coli*, a total kill being observed at a level of 20μg/plate. Taking into account the reduced sensitivity of RS112 to PQ compared with *E.coli*, a range of MC concentrations was chosen from 0 to 100μg MC/ml subculture. The upper concentration was limited by the commercial availability of MC (only supplied in 2mg lots), and its cost (ca. £10/mg).

The results obtained are shown in table 6.9 and figure 6.15. As for PQ, the results show a linear increase in *HIS*+ reversion with increasing MC concentration, and little or no induction of *ADE*+ reversion at the doses tested.

Table 6.9. The effect of Mitomycin C on *HIS*+ and *ADE*+ reversion in RS112.

MC in µg/ml	Generations	Survivors in %	*HIS*+/10^4 cells	rrf±SD (*HIS*+)	*ADE*+/10^5 cells	rrf±SD (*ADE*+)
0	5.04	100(1031)	1.63(1674)	1.00±0.02	3.69(3792)	1.00±0.04
25	5.23	89(1041)	2.24(2336)	1.38±0.03	3.67(3848)	0.99±0.04
50	5.21	86(1001)	2.65(2660)	1.63±0.07	4.04(4048)	1.09±0.02
100	4.86	100(963)	3.77(3635)	2.32±0.11	4.35(4192)	1.18±0.08

The level of toxicity observed at the highest dose was negligible, and ideally the experiment should be extended to concentrations of MC which show a higher toxicity, however, the preferential induction of DEL over ICR, in a linear, apparently thresholdless nature by MC is clear from these results.

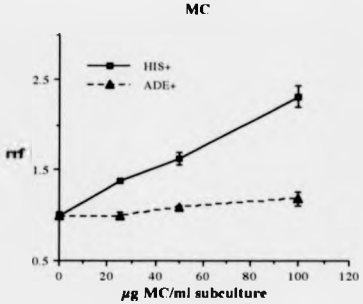


Figure 6.15. The effect of Mitomycin C on *HIS*+ and *ADE*+ reversion in RS112.

Holliday (1964) reported a 9 fold induction of mitotic gene conversion, between *trp*1 heteroalleles in a diploid strain of *S. cerevisiae* after treatment with 400μg/ml MC for 9.6hrs, indicating that higher doses of MC may induce *ADE*+ reversion in RS112.

Several other groups have also reported positive results with MC in yeast gene conversion assays (Zimmerman *et al.* 1984), although in each case, as for the results obtained with PQ, stationary phase cultures have been employed.

The dose response profiles of RS112 towards MC and PQ seemed to show a new kind of behaviour towards these oxidative mutagens, with a linear increase in DEL and no increase in *ADE*+ (at the same dose level). The reason for this dose response is unclear, but may be linked to the ability of PQ and MC to produce to produce O_2^- in a constant redox cycle,

and a preferential induction of DEL over ICR by these radicals (or their metabolites).

The next two chemicals tested were two chemical oxidants known to generate reactive oxygen species, phenyl hydrazine (PH) and cumene hydroperoxide (CH) (Levin *et al*, 1982; Levin *et al*, 1984) (figure 6.16).

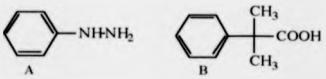


Figure 6.16. The chemical structures of A Phenyl hydrazine and B Cumene hydroperoxide.

These chemicals are both efficiently detected as mutagenic using the Ames strain TA102 at levels of 250μg/plate and 100μg/plate respectively.

Taking into account the reduced sensitivity of RS112 towards PQ and MC compared to the Ames tester strains, initial experiments with PH were performed using concentrations of up to 50mg/ml subculture. Surprisingly however, a 100% kill was observed at the lowest concentration (275μg/ml) and thus no data was obtained. Subsequent experiments were performed using reduced levels of PH, yielding the data in table 6.10 (see also figure 6.17).

Initial experiments with CH were performed using concentrations of 0, 5, 25, 50, 75, 100, 150, and 200μg CH/ml subculture. CH was added as a solution in DMSO (maximum amount DMSO added = 50μl) and thus, as a control, a 9th experiment was performed in which 50μl of DMSO alone was added to a subculture to check for any solvent induced effects on

recombination.

Table 6.10. Effect of Phenyl hydrazine on *HIS*+ and *ADE*+ reversion of RS112.

PH in µg/ml	Generations	Survivors in %	*HIS*+ /10^4 cells	rrf±SD (*HIS*+)	*ADE*+ /10^6 cells	rrf±SD (*ADE*+)
0	5.20	100(1267)	1.36(1718)	1.00±0.02	1.52(1924)	1.00±0.05
10	4.88	100(1316)	1.22(1585)	0.90±0.03	0.79(1035)	0.52±0.05
15	4.42	100(1285)	1.53(1472)	1.12±0.01	0.75(738)	0.49±0.03
20	4.52	65(512)	1.86(955)	1.37±0.17	0.57(441)	0.57±0.05
25	2.97	19(525)	4.43(2326)	3.26±0.08	0.47(245)	0.31±0.02
50	2.13	7(1060)	5.37(569)	3.95±0.24	0.46(49)	0.30±0.08

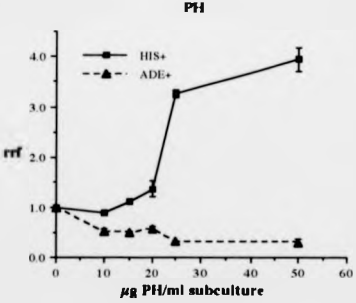


Figure 6.17. Effect of Phenyl hydrazine on *HIS*+ and *ADE*+ reversion of RS112.

Results from the initial experiments on CH showed that while little effect on either *HIS+* or *ADE+* reversion was observed at a concentration of CH of 25μg/ml (rrf *HIS+* 0.90±0.05, rrf *ADE+* 0.82±0.05), a total kill was observed at the next concentration (50μg/ml), no toxicity effects were observed at 25μg/ml. No major effects on cell survival or recombination were observed in the DMSO control (rrf *HIS+* 0.92±0.02, rrf *ADE+* 0.95±0.05) indicating that DMSO could safely be used as a solvent for potential recombinogenic chemicals up to a concentration of c. 5mg/ml subculture.

Further experiments were performed with a range of concentrations of CH up to 50μg/ml subculture. The following data was obtained (shown graphically in figure 6.18.).

Table 6.11. Effect of Cumene hydroperoxide on *ADE+* and *HIS+* reversion frequencies of RS112.

CH in μg/ml	Generations	Survivors in %	*HIS+*/10^4 cells	rrf±SD (*HIS+*)	*ADE+*/10^8 cells	rrf±SD (*ADE+*)
0	5.07	100(1058)	0.87(924)	1.00±0.03	3.40(360)	1.00±0.09
37	4.74	100(1090)	1.20(1308)	1.38±0.07	0.97(106)	0.29±0.06
40	4.66	100(1140)	1.17(1337)	1.34±0.07	1.18(135)	0.35±0.02
45	4.45	100(762)	1.31(1000)	1.50±0.08	1.75(133)	0.51±0.01
47	3.10	69(1848)	1.70(3093)	1.95±0.00	2.35(435)	0.69±0.02
50	1.16	30(2097)	3.35(713)	3.84±0.08	2.96(62)	0.87±0.02

The results obtained from the experiments using CH and PH were very exciting. The dose response profiles obtained in each case (figures 6.17, 6.18) were very similar to the dose responses obtained with the *non-*

mutagenic carcinogens, although maximum dose levels are much lower than those typically required for the induction of any effect by non-mutagenic carcinogens (doses for the non-mutagenic carcinogens are generally in mg/ml compared to μg/ml for the two chemical oxidants studied (Schiestl, 1989)).

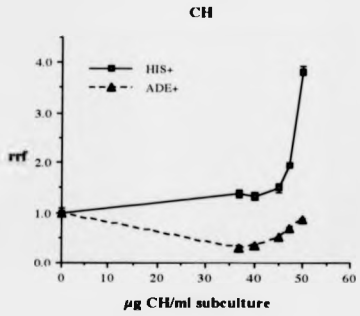


Figure 6.18. The effect of Cumene hydroperoxide on *ADE*+ and *HIS*+ reversion frequencies of RS112.

A further similarity in the dose response of RS112 to the two types of carcinogen is the association of the threshold concentration for DEL (*HIS*+) induction with the onset of toxicity. For both PH and CH the induction of DEL occurs at a concentration of chemical at which a significant toxic effect (both suppression of cell division and lethality) first becomes apparent. The induction of DEL by the non-mutagenic carcinogens is also generally associated with a toxic effect by the chemical (Schiestl *et al*, 1989).

Neither of the two oxidants tested here showed any induction

of ICR (*ADE+*), (CH has previously been reported as negative in mitotic recombination assays although in the same study *t-butyl* hydroperoxide was detected as positive; Callen and Larson, 1978), in fact both chemicals showed a significant *depression* of ICR at all doses tested (although in the case of CH, a partial recovery towards the baseline frequency was observed at the higher doses). This depression occurred at dose levels which had no toxic effect on the cultures and would thus seem to represent a real effect. Some small depression of ICR is often observed with the non-mutagenic carcinogens (Schiestl *et al*, 1989) and was observed in this work with aniline and paraquat, although not to the same extent as was observed with both PH and CH (c.70% depression in each case).

The final oxidative mutagen we wished to test on RS112 was hydrogen peroxide (H_2O_2). H_2O_2 is thought to generate highly reactive hydroxyl radicals (OH) *in vivo*, via the Fenton reaction(1), catalysed by Fe^{2+}.

$$(1)\ H_2O_2 + Fe^{2+} + H^+ \rightarrow OH + Fe^{3+} + H_2O$$

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

The role of OH^- in oxidative stress is of great interest. initial theories postulated the direct involvement of O_2^- in oxygen toxicity (McCord *et al*, 1971), however the low reactivity of O_2^- in aqueous solution led to speculation that it may mediate it's effects via OH^-, generated in the above reaction (Sawyer and Valentine, 1981; Michelson *et al*, 1977).

By investigating the effects of of mild oxidative stress on strains of *S. cerevisiae* deficient in either SOD or catalase, which remove O_2^- (2) and H_2O_2 (3) respectively, Bilinski *et al* (1985) concluded that, in yeast, only SOD had any important protective effect against oxidative stress.

(2) $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

(3) $H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$

Cellular catalases generally perform a peroxidative role in a variety of metabolic pathways, (DeDuve and Baudhin, 1966), reaction 3 occurs only at high H_2O_2 concentrations rarely reached under physiological conditions (Aebi and Wyss, 1978). H_2O_2 however is mutagenic to the Ames tester strain TA102 (Levin *et al*, 1984).

Initial experiments with H_2O_2 were performed with a range of concentrations similar to that which gave good results with cumene hydroperoxide, as it was expected that the two chemicals would behave in a similar fashion to each other. using a range of H_2O_2 concentrations up to 80μg/ml subculture the following results were obtained.

Table 6.12. Effect of H_2O_2 on *HIS*$^+$ and *ADE*$^+$ reversion of RS112.

H_2O_2 in μg/ml	Generations	Survivors in %	*HIS*$^+$ /10^8 cells	rrf±SD (*HIS*$^+$)	*ADE*$^+$ /10^8 cells	rrf±SD (*ADE*$^+$)
0	4.94	100(730)	1.38(1008)	1.00±0.01	3.95(288)	1.00±0.10
20	4.89	100(740)	2.72(2019)	1.97±0.06	12.3(908)	3.10±0.20
40	4.51	100(644)	3.28(2106)	2.38±0.08	28.0(1796)	7.08±0.31
60	1.73	74(686)	12.5(852)	9.05±0.40	49.5(2268)	12.5±0.47
70	0.82	53(236)	22.0(579)	15.9±0.65	39.5(692)	9.99±1.14
80	0.15	42(1299)	29.7(386)	21.5±0.42	45.2(391)	11.4±0.04

Also shown graphically in figure 6.19.

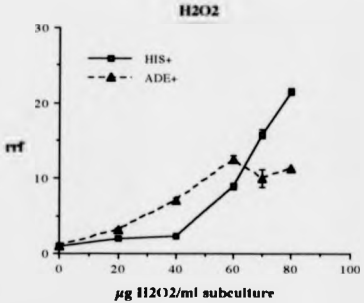


Figure 6.19. The effect of hydrogen peroxide on *HIS+* and *ADE+* reversion in RS112.

These results were at once exciting and intriguing. Both *HIS+* and *ADE+* reversion were efficiently induced by H_2O_2, however, while *ADE+* reversion was induced in a linear fashion with increasing dose (a plateau of response is apparent at doses which show high levels of toxicity), *HIS+* reversion is induced in a similar manner to that observed with the non-mutagenic carcinogens and with CH and PH. The threshold for a response in *HIS+* reversion once again corresponds with the onset of a toxic effect for the chemical, however the maximum level of induction obtained with H_2O_2 is higher than that observed with either PH, CH or with the majority of Ames negative carcinogens so far tested, the exceptions being formaldehyde and carbon tetrachloride (Schiestl *et al*, 1989).

It would seem likely that the different responses of RS112 to various oxidative mutagens reflect different types of radical species generated, or perhaps different modes of radical generation. For instance, the linear induction of DEL by paraquat and mitomycin C may reflect their ability to

generate O_2^- radicals in a continuous redox cycling, whereas the induction of DEL with an apparent threshold by other oxidants may reflect the generation of radical species as a purely 'last resort' defence mechanism after the over loading of other forms of defence. The induction of ICR by sub-lethal concentrations of H_2O_2 would seem to implicate hydroxyl radicals in this process, this would also seem to argue against a role for OH^- in the action of paraquat and mitomycin C (a *direct* involvement of O_2^-). The role of radical species in the induction of DEL and ICR is discussed in greater depth in Chapter 7.4.

6.2.4. Investigation into the Effects of two Hydroxyl Radical Scavengers on the Induction of DEL and ICR by Hydrogen Peroxide.

The involvement of oxygen radicals in a chemical process may be inferred from the ability of 'scavengers' of radicals to inhibit the process. Many such scavengers are known. These include naturally occurring antioxidants such as α-tocopherol (vitamin E), which is thought to be important in protecting against lipid peroxidation *in vivo* by intercepting aqueous phase radicals before they can damage cellular membranes (Simic, 1983), and chemical antioxidants. Commonly used chemical radical scavengers include DMSO, thiourea (TU), tetra methyl urea (TMU), ethylene glycol and ethanol. All of these chemicals inhibit the natural activity of human killer cells (Suthanthiran *et al*, 1984). Cytotoxicity of these cells is thought to be due to the production of OH^- radicals from the lipoxygenase pathway of arachidonic acid metabolism (*ibid*). There are many other examples of biological reactions being inhibited by scavengers of radical species (see Greenwald and Cohen, 1983).

Due to the shortage of time remaining for the completion of the

project, it was decided to to investigate the effects of two reported OH radical scavengers, DMSO and tetramethyl urea, on the induction of DEL and ICR by the oxidative mutagen H_2O_2. Thiourea and α–tocopherol were also considered as radical scavengers, however, thiourea is an efficient inducer of both DEL and ICR (Schiestl *et al*, 1989) and α–tocopherol is not sufficiently soluble in aqueous media.

The first experiment performed was the incubation of RS112 with a set level of H_2O_2 of 60μg/ml subculture (a level known to give good induction of DEL and ICR) and a range of DMSO concentrations up to 40mg/ml (an approximate 300× molar excess over the oxidant). In all experiments, controls were performed to check for any recombinogenic effect of the scavenger. Rrf's for subcultures containing both oxidant and scavenger were calculated using the 'scavenger only' control as the baseline.

The results obtained in this initial experiment are given in tables 6.13 and 6.14 and graphically in figures 6.20 and 6.21.

The results show that, at the doses used, DMSO causes some induction of both DEL and ICR. The effect of DMSO on the induction of DEL by H_2O_2 is not very clear cut from these results. There is an apparent reduction in effect at all the doses (≈ 40% at the highest dose), however, when the increase in DEL caused by DMSO is ignored, there is no overall decrease in effect, in fact a small increase in effect is seen (rrf (+DMSO/-DMSO) = 5.99 @ +/-10mg/ml DMSO, 60μg/ml H_2O_2).

Table 6.13. The effect of DMSO on the induction of *HIS*+ reversion in RS112 by 60μg/ml H_2O_2.

DMSO in mg/ml	Generations		Survivors in %		*HIS*+ /10^4 cells		rrf±SD (*HIS*+)	
	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2
0	4.87	3.27	100	59	1.20	5.92	1±0.08	4.93±0.15
10	4.95	2.83	100	58	1.93	7.19	1.61±0.06	3.72±0.20
20	4.67	2.55	100	74	1.85	6.40	1.54±0.03	3.46±0.13
40	4.67	2.64	100	59	2.00	6.01	1.66±0.06	3.01±0.18

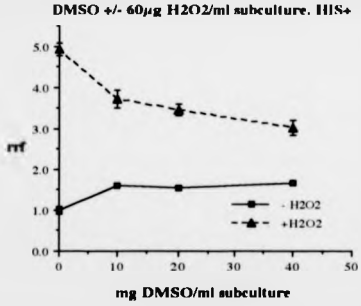


Figure 6.20. The effect of DMSO on the induction of *HIS*+ reversion by 60μg/ml H_2O_2.

Table 6.14. The effect of DMSO on the induction of *ADE*+ reversion in RS112 by 60μg/ml H_2O_2.

DMSO in mg/ml	Generations		Survivors in %		*ADE*+ /10^5 cells		rrf±SD (*ADE*+)	
	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2
0	4.87	3.27	100	59	1.56	39.2	1.00±0.07	25.1±1.36
10	4.95	2.83	100	58	3.40	33.4	2.18±0.31	9.82±0.70
20	4.67	2.55	100	74	2.07	20.6	1.32±0.12	9.95±1.06
40	4.67	2.64	100	59	1.59	15.5	1.02±0.	9.75±0.76

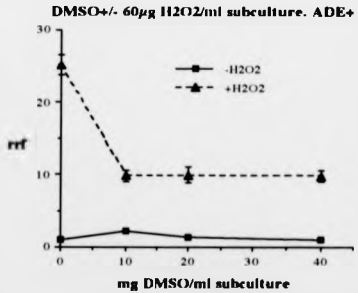


Figure 6.21. The effect of DMSO on the induction of *ADE*+ reversion by 60μg/ml H_2O_2.

The results for ICR are much clearer cut than those for DEL, even without taking into account the slight induction of ICR by DMSO (more evident at the lowest dose than at the higher two doses) there is a significant reduction in the ICR inducing effect of H_2O_2 (approximately 60%) at each dose.

It was decided to perform a second experiment in which the concentration of DMSO would be kept constant at 30mg/ml subculture, while the concentration of H_2O_2 was varied between 0 and 85μg/ml. In the previous experiment, a plateau in the effect of DMSO was apparent at levels greater than 10mg/ml and so this concentration was chosen to give a good inhibitory effect with a 'safety margin' for the increase in H_2O_2 concentrations used. The results are shown in tables 6.15 and 6.16 and in figures 6.22 and 6.23.

Table 6.15. The effect of 30mg DMSO/ml subculture on the induction of *HIS+* reversion in RS112 by H_2O_2.

H_2O_2 in mg/ml	Generations		Survivors in %		*HIS+* /10^4 cells		rrf±SD (*HIS+*)	
	-DMSO	+DMSO	-DMSO	+DMSO	-DMSO	+DMSO	-DMSO	+DMSO
0	4.92	5.08	100	100	1.07	1.71	1.00±0.06	1.00±0.04
25	4.71	5.14	100	100	1.62	2.29	1.52±0.02	1.32±0.03
50	2.84	3.19	80	64	4.79	4.64	4.47±0.16	2.71±0.18
75	0.16	0.21	72	65	20.6	12.4	19.3±1.08	7.28±0.14
85	0.53	0.26	48	54	20.4	13.3	19.1±1.55	7.80±0.06

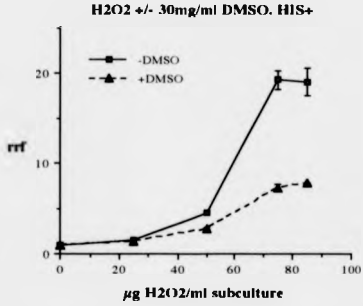


Figure 6.22. The effect of 30mg DMSO/ml subculture on the induction of *HIS*+ reversion in RS112 by H_2O_2.

Table 6.16. The effect of 30mg DMSO/ml subculture on the induction of *ADE*+ reversion in RS112 by H_2O_2.

H_2O_2 in mg/ml	Generations		Survivors in %		*ADE*+ /10^5 cells		rrf±SD (*ADE*+)	
	-DMSO	+DMSO	-DMSO	+DMSO	-DMSO	+DMSO	-DMSO	+DMSO
0	4.92	5.08	100	100	15.6	14.4	1.00±0.06	1.00±0.06
25	4.71	5.14	100	100	17.9	8.91	1.15±0.07	0.62±0.03
50	2.84	3.19	80	64	35.5	13.6	2.27±0.14	0.95±0.03
75	0.16	0.21	72	65	36.4	33.2	3.64±0.32	2.31±0.17
85	0.53	0.26	48	54	39.8	35.8	3.98±0.65	2.49±0.16

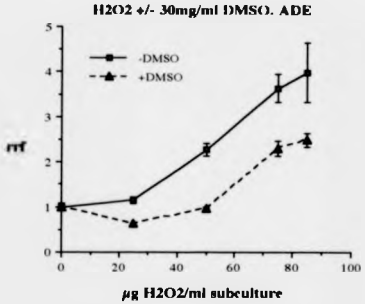


Figure 6.23. The effect of 30mg DMSO/ml subculture on the induction of *ADE+* reversion in RS112 by H_2O_2.

It is clear from these results that DMSO significantly reduces the induction of both DEL and ICR by H_2O_2. The lower level of ICR induction in this experiment compared with the previous two experiments was probably due to an unusually high base level of *ADE+* revertants, possibly due to the use of an 'older' colony to seed the starter culture.

In the case of DEL, the inhibition is most evident, though not complete, at the highest doses of H_2O_2, this explains the relatively small effects observed in the previous experiment.

DMSO, at this dose, completely inhibits the induction of ICR at the lowest two doses of H_2O_2, although the levels of ICR rise in a parallel fashion to the -DMSO control at the next two doses, (*i.e.* the protective

effects of DMSO appear to have been overwhelmed by very high levels of oxidant).

The incomplete protection by DMSO may reflect a saturation of antioxidant effect by high H_2O_2 levels, however if this were the case then increasing the levels of oxidant further would be expected to result in an increase in the levels of recombination to those seen in the absence of antioxidant. This is not observed in the case of DEL, in fact the level of DEL induction reaches a plateau at the same H_2O_2 concentration in the presence and absence of DMSO, it is possible therefore that these observations reflect differential protection by DMSO against different radical species, *i.e.* efficient protection against one type of radical (OH ?) and inefficient protection against others (O_2^-?). Some evidence of a plateau is also observed in the data for *ADE+* reversion. It is, however, possible that the plateau of induction is due to the high levels of cell kill observed at the highest two doses, DMSO apparently providing little or no defence against the toxic effects of H_2O_2.

The second antioxidant we wished to test was TMU. Referring to the observations of Suthanthiran *et al* (1984) who used TMU at concentrations of 10-25mM, the antioxidant was initially tested, as in the first experiments with DMSO, with a set concentration of H_2O_2 (in this case 50μg/ml) and a range of TMU concentrations upto 2.5 mg/ml (21.5mM). The use of higher concentrations of TMU was hindered due to its tendency to attack the plastic of the culture vessels. The results are presented in tables 6.17 and 6.18 and in figures 6.24 and 6.25.

Table 6.17. The effect of TMU on the induction of *HIS+* reversion in RS112 by 50μg/ml H_2O_2.

TMU in mg/ml	Generations		Survivors in %		*HIS+* /10^4 cells		rrf±SD (*HIS+*)	
	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2
0	5.19	4.65	100	100	1.65	7.38	1±0.05	4.47±0.12
0.4	5.25	4.30	93	100	1.71	7.58	1.03±0.04	4.43±0.19
0.8	4.84	4.70	100	100	1.57	6.36	0.95±0.04	4.05±0.07
2.5	4.98	4.39	100	100	1.92	7.08	1.17±0.11	3.69±0.11

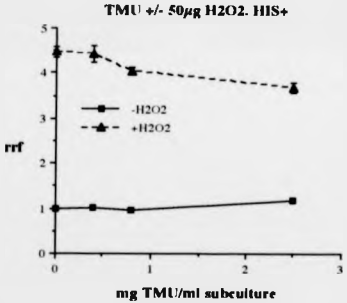


Figure 6.24. The effect of TMU on the induction of *HIS+* reversion in RS112 by 50μg/ml H_2O_2.

Table 6.18. The effect of TMU on the induction of *ADE*+ reversion in RS112 by 50μg/ml H_2O_2.

TMU in mg/ml	Generations		Survivors in % *ADE*+ /10^5 cells		rrf±SD (*ADE*+)			
	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2	-H_2O_2	+H_2O_2
0	5.19	4.65	100	100	0.559	41.6	1±0.25	74.3±5.18
0.4	5.25	4.30	93	100	0.734	46.3	1.31±0.03	63.1±1.28
0.8	4.84	4.70	100	100	0.738	34.8	1.32±0.30	47.2±2.62
2.5	4.98	4.39	100	100	0.948	27.9	1.70±0.11	29.4±1.36

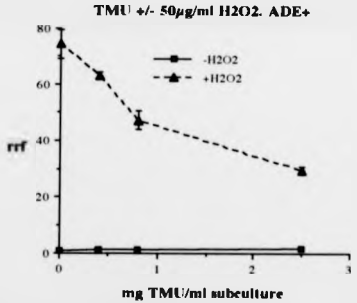


Figure 6.25. The effect of TMU on the induction of *ADE*+ reversion in RS112 by 50μg/ml H_2O_2.

Results obtained in experiments with TMU parallelled those observed with DMSO in the equivalent experiment. Only a very small reduction in the level of DEL induction was observed and this is negligible when the (very slight) induction of DEL by TMU is ignored, however, once again the level of induction by H_2O_2 is relatively small and a larger effect by TMU may be observed at higher H_2O_2 doses.

In contrast, the induction of ICR by H_2O_2 (which is slightly higher than usual, probably due to the unusually low levels of spontaneous reversion), is significantly moderated by TMU at each level, with a maximum 61% reduction at the highest dose of TMU. Once again the induction is not completely inhibited, however, the inhibiting effects of TMU do not appear to be saturated at the highest dose and a further inhibition of H_2O_2 mediated ICR may be possible at higher doses.

Due to a lack of time available, it was, unfortunately, not possible to study the effect of a constant level of TMU on the induction of DEL and ICR by a range of H_2O_2 concentrations, as had been done with DMSO. Intuitively, the results would be expected to be similar.

CHAPTER 7: DISCUSSION.

7.1. Studies Employing MC4100 Mud1(Apr, *lac*) and UWE103(pBR322).

7.1.1. General Comments.

Studies on the two existing transposition tester strains revealed a number of problems with the procedure. Most notable amongst these was an irreproducibility of apparent small changes in tf between experiments and a variation in the level of effect observed with the major transposogens (MNNG, heat induction) between experiments (although the inductive effect itself was always reproducible). Some of the reason for these observations must undoubtedly lie in variations in cell culture densities and baseline tf's prior to treatment as these were not standardised. Some variation in tf is unavoidable due to the unpredictable nature of the event. Wilkins (1987) reported a 5 fold reduction in apparent tf of Mud1(Apr, *lac*) as the density the cell culture plated out increased from ~ $0.5\text{-}4\times10^9$ cells/ml, this was cited as further evidence of a role for cGMP in transposition (cGMP levels being higher in rapidly growing cells). Intuitively this observation is impossible although the same trend in effect was observed in the course of this work. Any transposition event occurring early during culture growth and giving rise to a *lac*+ cell, would also produce *lac*+ progeny, thus, transposition frequency would *increase* rather than decrease during the increase in cell density, due to the continued rise in the number of cells able, by newly acquired transposition products, to produce *lac*+ progeny, though this change would be small due to the rarity of transposition. Two experiments not reported in the main results section were performed to investigate further. In the first, cells from a single overnight culture of MC4100 Mud1(Apr, *lac*) were plated at a range of

densities onto M63 lactose plates, estimation of total cell numbers was performed by plating 3×100μl of a 10^6 dilution of the original overnight culture on M63 glucose plates. Apparent tf's were calculated for each plating density after 72 hours incubation at 30°C (figure 7.1.)

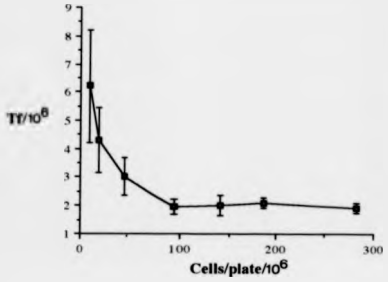


Figure 7.1. Graph of number of cells per plate vs. apparent tf, showing the effect of plating density on the apparent tf of Mud1(Apr, *lac*). Error bars indicate the standard deviation in tf (three plates). Raw data not given.

The results of the first experiment show the same trend reported by Wilkins over the course of many experiments and indicate that it is the number of cells on the plate and not the density of the original culture which affects the apparent tf. A plateau of apparent tf is obtained, probably representing the 'true' tf (in this case ~ 2×10-6) the lower limit of this plateau represents about 100 *lac*+ colonies/plate and the highest value about 600. These results were confirmed in the second experiment where cells were taken

from an exponentially growing culture at culture densities between 1.54×10^8 and 3.87×10^9 cells/ml (mid to late log phase) and plated, at an approximately equal number of cells/plate ~ 2×10^8. The results (not shown) indicated no variation in baseline tf ($2.05\times10^{-6}\pm 0.29\times10^{-6}$) between the culture densities.

Increased tf at low plating densities probably indicates an increased background growth of *lac*⁻ cells on trace amounts of utilisable carbon sources in the medium (decreased competition for resources) leading to an increased likelihood of *lac*⁺ inducing transpositions while on the plate.

A need to standardise the procedures so that equal numbers of cells are plated in tf experiments is evident. This is easily achieved by using overnight cultures grown to equal cell densities in each experiment. This would ensure more reliable baseline tf's, and also a constant molar ratio of cell to chemical in transposogenicity tests. Cell densities of subculture experiments should also be standardised for the same reasons.

Another problem with the assays is the variation in intra-experimental results (as evidenced by SD values). In this research project, SD values were generally much higher than previously reported (Wilkins, 1987), and may have masked very small differences in tf values in response to chemicals. Statistical analysis, using the students T test, was by Wilkins used to confirm the significance of small (~ ±30% of baseline) effects on tf. No way of reducing the observed SD's is immediately evident, similar (usually higher) SD's were observed by other workers using the same systems and variations are assumed to be due to generally unavoidable systematic errors. This would indicate that interpretation of small variations in tf as significant must be done with much attention, not just to intra-experimental SD and statistical analysis,

but to reproducibility of the result between experiments. Small variations in effect (< 2 fold changes in frequency of observed effect) are not suitable for short term tests, which must give results reproducible between laboratories and laboratory workers as well as between experiments to serve any useful purpose.

7.1.2. Observations Made During Investigations into the Role of cGMP in Transposition.

As outlined in chapter 1.3, a variety of observations led to the proposal of a role for cGMP in transposition of both Mud1(Apr, *lac*) and Tn5 by Gary Wilkins (Wilkins, 1987; Wilkins and Swoboda, 1987). No convincing explanation for the similar level of response of both transposition systems to raised cGMP levels was proposed and it was decided that any cGMP mediated effect must occur via a different mechanism in each case.

The results of experiments reported in this work do not support the case for cGMP involvement in transposition of either Mud1(Apr, *lac*) or Tn5. No enhancement of tf is observed with dbcGMP levels up to 10 times higher than those reported by Wilkins (*ibid*) to have an elevating effect on tf of both transposons. The increase in tf observed with MNNG is not augmented in the presence of the cGMP phosphodiesterase inhibitor M&B 22948, nor does the powerful stimulator of guanylate cyclase SNP have any effect. This would seem to argue against an NO mediated stimulation of cyclase activity as the cause tf enhancement by MNNG.

Enhancement of tf by MNNG does appear, however, to be largely epigenetic in origin. Later studies with *recA*- and Ames test compatible tester strains indicate that mutagenicity and transposogenicity of the chemical

occur at very different concentrations. The nature of the transposogenic signal in response to nitro-nitrosoguanidines remains ambiguous. The lag in onset of the effect observed in short period subculture experiments is suggestive of a requirement for protein synthesis in the response. An investigation into the nature of the proteins being synthesised in response to NNG treatment would undoubtedly shed more light onto the mechanism of their action. these will include SOS encoded proteins and the transposase protein but may include another, possibly host-encoded, protein synthesised in response to exposure of the cell to NNG's, responsible for the induction of transposition. This type of study was beyond the scope of this research project and represents a project on its own. An elucidation of the mechanism of the MNNG effect is of great interest however, and may give more insight into the mechanisms of transposition and its control.

A plateau in the level of tf induction by MNNG, after less than 30 minutes exposure to the chemical, was also apparent from the short period subculture experiments. This may be due to the exhaustion of MNNG by bacterial metabolisation or may be due to the exhaustion of a second factor necessary for the production of the ultimate transposogenic species. Identification of a possible 'accessory factor' in the transposogenicity of MNNG would also give valuable information as to the nature of the the (so far) uniquely high transposogenicity of the nitro nitrosoguanines.

The apparent large depression of transposition by TEA, specific to Tn5, is also of interest. The observation requires conformation in a different assay system, *i.e.* the subculture/mating out system, in order to rule out a systematic effect (*e.g.* the increased solublisation of Km in the 1mg/ml Km plates increasing intracellular levels of the antibiotic to inhibitory levels). If the

effect of TEA is a specific *anti*-transposogenic effect on Tn5, an elucidation of the mechanism of this effect is of great importance in understanding the mechanism of control of Tn5 transposition. A fundamental difference in control of the two transposons is also suggested.

7.1.3. The Temperature Sensitivity of Mud1(Apr, *lac*).

The observed effect of incubation at 37°C on Mud1(Apr, *lac*) is almost certainly due to heat inactivation of the temperature sensitive Mu c protein (the Mu repressor). The c protein binds to the same sequences on the Mu genome as the Mu A (transposase) protein, thus blocking A-mediated transposition of the prophage (see chapter 1.3).

Inactivation of the temperature sensitive c protein (cts) is most likely caused by an inactivating change in conformation at the raised temperature, however, the involvement of a host-encoded function in the inactivation cannot be ruled out without further study. This possible host function may be present at raised levels at the elevated temperature. *E. coli* is well adapted to growth at 37°C, however, a sudden elevation to 37°C following previous growth at 30°C may induce a heat shock response. Heat shock results in the synthesis of more than 20 proteins at elevated levels, one of these (*e.g.* the *lon* protease) may be responsible for c protein inactivation. The lag phase in heat induced effect on Mud1(Apr, *lac*) transposition suggests *de novo* protein synthesis, although this may be solely due to the requirement for Mu *A* gene expression for transposition. Alternatively, a normal cell constituent may interact with the c protein exclusively at the higher temperature yeilding an inactive repressor complex.

7.1.4. The Effects of Oxidative Stress on the Transposition of Mud1(Ap^r, *lac*) and Tn5.

Following the observation of increased tf of Mud1(Ap^r, *lac*) in response to extracellularly generated O_2^- (Wilkins, 1987), it was anticipated that the generation of high *intra*cellular levels of superoxide radical by the redox-active compounds paraquat and mitomycin C, would be highly transposogenic (bacterial membranes being generally impermeable to radical species).

Free oxygen radicals are implicated in a wide range of diseases, including carcinogenesis. The tumour promoting phorbol esters are thought to raise intracellular levels of free oxygen radicals via a stimulation of arachidonic acid metabolism (Marx, 1983). Oxygen radicals cause DNA strand scission, backbone breakage and base liberation and chemical oxidants have been detected as mutagenic using specially constructed Ames tester strains of *S. typhimurium* (Levin *et al*, 1984).

The possible link between high levels of oxygen free radicals and large scale alterations in DNA structure has been discussed (Marx, 1983; see also chapter 1.2) and it would therefore be desirable for any short-term test based on such large scale changes to be able to reflect this link.

The results obtained employing the two oxidative mutagens PQ and MC in the two existing transposition tester strains indicated that, although some small increases in tf were evident in response to the chemicals, no major transposogenic effects were detectable over the levels previously reported for various conventional mutagens (Wilkins, 1987).

7.2. The Development of New Transposition Tester Strains.

7.2.1. The Development of a Mating Out Assay for Transposition.

A new, simple assay for transposition was developed, based on the detection of transposition of Tn5 into a transmissible plasmid by the acquisition of Km resistance in a Km sensitive strain following mating with the transposition tester strain. The system, which involves pre-incubation of the tester strain with the chemical to be tested, in subculture, proved to give good reproducibilities between duplicates of the same experiment and scoring of tf's was simplified by the definite nature of the observed endpoint (Km resistance).

The test proved responsive to the transposogenic effects of MNNG although the sensitivity of the test (maximum level of tf induction) was comparable to that of the existing tests to treatment with MNNG in subculture, *i.e.* about half that observed in plate incorporation assays using UWE103(pPH1J1). The decreased sensitivity to MNNG in subculture may be due to the exclusion of glucose from the subculture medium (unlikely as the plate incorporation assays for Mud1(Ap^r, *lac*) employ *lactose* as the carbon source), the exhaustion of MNNG by bacterial metabolism, or due to the exhaustion of an 'accessory factor' (see 7.1.2) which is gradually replenished to undergo further rounds of transposogenesis. A further possibility is that the addition of MNNG to top agar, along with the cells, results in exposure of the cells to higher concentrations of MNNG for a short time, before diffusion of the chemical throughout the agar plate occurs. This short exposure to high concentrations may be transposogenic while avoiding the major toxic effects.

The new, mating out test system is easily adaptable to the study

of Mud1(Ap^r, *lac*) transposition, by the simple transformation of the existing MC4100 Mud1(Ap^r, *lac*) strain with (pPH1J1) using FE20(pPH1J1) as a donor. This has, in fact, already been done for a different reason (chapter 5.2.2). A simple conformation of the genotype of the strain MC4100 Mud1(Ap^r, *lac*)(pPH1J1) and adaption of the test system (detection of FE20(pPH1J1)::Mud1(Ap^r, *lac*) on LB/Nal/Gm/Ap plates) will allow the study of transposition of Mud1(Ap^r, *lac*) in a system directly comparable to that used for Tn5.

The potential for the facile expansion of the test to different transposons, perhaps in other bacteria (*e.g. Salmonella*) also exists.

7.2.2. Study of the Effects of two Types of Host Cell Mutation on the Transposition of Tn5.

Transposition of Tn5 in *recA*$^-$ and *dam*$^-$, *dcm*$^-$ derivatives of the tester strain UWE110(pPH1J1) was studied.

Transposition is widely accepted to be independent of the host SOS/recombinational repair system controlled by the *recA* gene. However, the possibility existed that the induction of tf by transposogens such as MNNG was either *recA* dependent (mediated by an SOS function) (*recA*$^-$ strains would therefore be insensitive to transposogens), or that the transposogenic signal was related to the type of DNA damage repaired by SOS genes (*recA*$^-$ strains would thus be more susceptible to transposogenesis by mutagenic agents).

The transduction of UWE110(pPH1J1) to *recA*$^-$ resulted in a tester strain 10-50 times more sensitive to the lethal effects of MNNG without a corresponding increase in sensitivity to its transposogenic effects. It was

unclear whether the lack of tf induction by MNNG was due to a *recA* dependency of the effect or due to the sensitivity of the *recA*- strain to cell kill by MNNG at concentrations which would normally induce transposition via a non-mutagenic pathway. A recent study by Kuan *et al* (1991) has shown an elevated frequency of Tn5 transposition in a host with a mutation resulting in the constitutive activation of the recA protease activity. The Tn5 inhibitor may therefore be susceptible to cleavage by activated recA protease, or the Tn5 promoter sequence may include a binding site for the lexA inhibitor protein (see figure 4.1 for an explanation of the SOS inducible repair system). Alternatively, recA protease may form an active Tn5 transposase by cleavage of an inactive (or less active) precursor protein.

The effect of the *dam-, dcm-* mutation was to raise the basal level of transposition of Tn5 5-50 fold, due to the reduction in transcriptional control exerted by methylation of adenine bases at methylation motifs (GATC) in the Tn5 promoter sequences (IS50R) (Yin *et al*, 1988). There was no equivalent change in sensitivity to the transposogenic effect of MNNG, however the increased baseline tf of the strain will make it more suitable to the study of tf depressing chemicals such as TEA.

7.2.3. The development of Transposition Tester Strains in Ames *Salmonella* Mutagenicity Tester Strains.

After several abortive attempts, lysogens of Mud1(Ap^r, *lac*) in three strains of *S. typhimurium*, developed for use in the Ames test for mutagens, were constructed. Preliminary results indicated that Mud1(Ap^r, *lac*) transposes in all three strains to give *lac*+ phenotypes. Apparent tf in a strain deficient in the enzyme pathway responsible for excision repair (*uvrB*) was

higher than in the equivalent *uvrB*+ strain. This observation may have been due to an underestimation of tf in the *uvrB*+ strain due to a reduced growth rate of *lac*+ colonies, possibly caused by a reduced activity of lactose permease in the strain, which has a defect in the pathways of cell wall biosynthesis (*rfa*) leading to an altered outer membrane structure (Maron and Ames, 1983).

In preliminary experiments, neither of the strains tested showed a level of sensitivity to the transposogenic effects of MNNG equal to that observed in *E. coli* MudI(Ap^r, *lac*) lysogens. The maximum level of MNNG induction in the *rfa* strain was observed at an MNNG concentration approximately 5 times lower than in an *E. coli* host, probably due to the increased membrane permeability of the *rfa* strain.

The presence of the *uvrB* mutation in TA1950 MudI(Ap^r, *lac*), which increases the sensitivity of the strain to mutagens (*ibid*) did not appear to increase sensitivity of the strain towards the transposogenic effects of MNNG. This, and the observation that mutagenic and transposogenic effects occur at different concentrations of the chemical, argue against any link between mutagenicity and transposogenicity and suggest an epigenetic mechanism of NNG induction of transposition.

7.3. Overview of Results Obtained in Transposition Tests and Proposals for Future Research.

In view of the ultimate aim of the project, the development of a novel, short-term test for carcinogens, based on the detection of gross changes in DNA structure, the results obtained during studies on bacterial transposition were disappointing and would seem to indicate that transposition was not

likely to be a suitable model for carcinogen induced genetic rearrangement.

The insensitivity of transposons to stimulation by external influences, such as treatment with mutagenic and other chemicals, is well documented. There are some examples of such influences, however. In addition to the effect of MNNG on Mud1(Apr, *lac*) and Tn5 first described by Swoboda and Hayer, the transposon Tn917, which encodes resistance to the macrolide antibiotic erythromycin A in *Streptococcus faecalis*, shows an increased transposition frequency in the presence of erythromycin (Tomich *et al*, 1980). Similarly, the Hg resistance encoding transposon Tn501 shows elevated tf's in the presence of Hg^{2+} ions (Arthur *et al*, 1981). Datta *et al* have reported an increase in tf of Tn9 in response to a variety of chemicals including acetate and DMSO, conspicuously, MNNG and other mutagens had no effect. An involvement of cell membranes with transposition was hypothesised.

Thus, it would appear that where chemical influences on transposition are observed, they are not generally related to any carcinogenic potential. This is further indication of the unsuitability of a transposition based system as a suitable model for carcinogen induced genetic rearrangement.

The results do, however, point to several possible experiments which may increase understanding of the mechanisms of transposition and their control.

Elucidation of the mechanism of MNNG elevation of tf is of prime interest, assuming that a nitroxide radical mediated elevation of cGMP levels is not involved. The possible requirement of an 'accessory factor' proposed, following the observation of a saturation in MNNG induction of tf after only a short exposure to the chemical, could be easily tested by the

addition of a further dose of MNNG to subcultures previously incubated in the presence of the chemical for 1-2 hours. If the plateau in time-dependent response is due to a depletion of MNNG, then it should be further enhanced by the addition of more chemical. If another factor is required then it may be possible to identify this by supplementation of the subculture with possible candidates. A likely candidate for any accessory factor would be intracellular thiol levels. MNNG is activated by cysteine and other thiols *in vivo* to generate its alkylating and nitroxide radical generating intermediates (figure 7.2.).

$$CH_3.N(NO)C(NH).NH.NO_2 + RSH \rightarrow CH_3N^+{=}N\ \ OH\ + RS.C(NH)NH.NO_2$$

OR

$$\rightarrow CH_3N{:}C(NH_2).NH.NO_2 + RS{-}NO$$

Figure 7.2. The reaction of MNNG with a thiol to give either diazomethane or S-nitrosothiol.

Also of interest is the identification of proteins synthesised in response to MNNG treatment. These will undoubtedly include SOS encoded proteins and probably the respective transposase proteins of the two transposons, but may also include a specific NNG response protein responsible for this class of chemical's transposogenicity.

Of equal interest to the mechanism of transposogenicity of MNNG, is the conformation and characterisation of an *anti*-transposogenic effect on Tn5 by TEA. Confirmation of the effect could easily be achieved by incubation of UWE103(pBR322), with the solvent, in liquid subcultures.

Further confirmation from studies with the newer test strains UWE110(pPH1J1) and UWE103(pPH1J1) would give conclusive proof of any reduction in tf.

Transduction of the new transposition tester strains UWE110(pPH1J1) and UWE103(pPH1J1) with Mud1(Apr, *lac*) would provide strains capable of comparing the transposition of Mud1(Apr, *lac*) and Tn5 directly, using the same basic assay system. Further transduction of the *dam*-, *dcm*- strain GM48(pPH1J1) and the *recA*- strain UWE110 *recA*- (pPH1J1) RI would provide strains capable of investigating the effects of these two mutations on the transposition of Mud1(Apr, *lac*).

7.4. Results Obtained using the Yeast Based Assay for Mutagenic and Non-mutagenic Carcinogens.

7.4.1. General Comments.

The original development of this assay system, by Schiestl and co-workers (Schiestl *et al* 1989), was undertaken with the aim of producing an assay for gross changes in DNA structure caused by carcinogens, which would be able to detect carcinogens which do not have any mutagenic activity (as defined by their ability to revert any of the Ames tester strains to histidine prototrophy), and may be missed by existing short-term tests. These were the same aims as those expressed in the introduction of this manuscript in the investigation of possible transposition based assays.

Initial results obtained using the yeast strain RS112 (Schiestl, 1989; Schiestl *et al*, 1989) indicated that it showed very considerable promise as a potential test for carcinogens. Both mutagenic and 'non-mutagenic'

carcinogens being detected as recombinogenic in the organism. Furthermore a difference in the modes of action of the two types of chemical was reflected by a difference in the type of recombination induced, conventional mutagens inducing both inter- and intrachromosomal recombination with equal efficiency, whereas non-mutagenic carcinogens induced only intrachromosomal recombination (for a detailed description of the system see chapter 6.1).

The reproducibility of the results was proved by reproduction of a small, representative sample during the course of this work. Additionally the levels of effect observed were greater than 2 fold for all of the non-mutagenic carcinogens so far studied (levels of effect for mutagenic carcinogens were generally considerably higher) (*ibid*).

A steady level of baseline recombination frequency, between experiments, in the DEL (intrachromosomal) recombination assay, is ensured by the inclusion of a counter-selecting factor in the assay (*i.e.* the acquisition of a leucine requirement following a DEL event, resulting in an inability of HIS+ revertants to divide and accumulate prior to plating). Low baseline frequencies of ICR however, are dependant on the seeding of initial cultures from fresh (< 24 hours old) colonies, and avoiding the depletion of adenine in the medium by overgrowth prior to subculturing. Variations in the level of ICR induction, apparently relating to the initial proportion of ADE+ revertants, was evident during the course of this study.

In general, effects on recombination, in response to chemical treatment, observed using RS112 were significantly larger and more reproducible than any observed effects on transposition frequencies.

7.4.2. The Effects of Chemically Induced Oxidative Stress on DEL and ICR in RS112.

The results obtained with the redox active compounds paraquat and mitomycin C, which had shown small transposogenic effects, indicate that, as predicted, the damage induced by superoxide (O_2^-) radicals is highly recombinogenic, however the induction of recombination is specific for DEL under the experimental conditions employed. The ultimate recombinogenic species is ambiguous, a direct attack of O_2^- on DNA or an effect mediated by the production of other, more active radicals such as the hydroxyl radical (OH) may be involved. The apparent lack of a threshold in the response may reflect the constant redox cycle generation of the superoxide radical.

The similarities in the dose response profiles of the oxidative mutagens phenyl hydrazine and cumene hydroperoxide in the DEL and ICR assays, to those observed with the non-mutagenic carcinogens, is of extreme interest. CH may generate alkoxy radicals *in vivo* via the Fenton reaction with Fe^{2+}, in an analogous fashion to the production of hydroxyl radical by H_2O_2. The dose response profile of H_2O_2 itself, however, showed a different type of effect, with high levels of induction of both DEL and ICR. DEL was induced in a fashion reflecting the profile of induction by aniline, $CdCl_2$ and the two oxidative mutagens tested, whereas ICR, which was not induced by any of the other oxidants investigated, was induced in a typically 'mutagenic' fashion. The reason for these differences is unclear but may reflect the generation of different radical species by OH and RO radicals in subsequent reactions.

The inhibition of H_2O_2 induced DEL and ICR by DMSO directly

implicates the hydroxyl radical in the effect. DMSO is highly soluble in biomaterials and is an extremely efficient scavenger of hydroxyl radicals (i) (Simic and Taylor, 1988).

(i) $(CH_3)_2SO + OH \rightarrow (OH)(CH_3)SO + CH_3$

The replacement of OH^- with a methyl radical in this reaction, which is subsequently reduced to methane by addition of a proton, probably abstracted from glutathione *in vivo*, may explain the incomplete inhibition of the H_2O_2 induction of DEL and ICR.

The inhibition of effect by DMSO does not, however, prove that OH^- reacts directly with DNA to induce recombination. In biological systems free radical cascades occur naturally and identification of the particular radical species responsible for a given type of biomolecular damage is difficult.

Cadmium chloride is a known clastogenic agent and its ability to induce chromosomal aberrations and DNA strand breaks has been shown to be inhibited by superoxide dismutase, catalase and scavengers of radical species (Biggart and Murphy Jr., 1988). $CdCl_3$ induced damage may therefore be mediated by oxygen free radicals. Interestingly, oxidative damage due to $CdCl_3$ is thought to have induced a 300bp *deletion* in a virally encoded oncogene, isolated following treatment of rat kidney 6m2 cells infected with the murine sarcoma virus MuSVts110 (*ibid*) with the metal salt.

It is tempting, therefore, to speculate that the induction of DEL by this, and some of the other'non-mutagenic' carcinogens, reflects the formation of reactive radical species by high concentrations of the chemical. The characteristic lag in DEL induction may reflect an efficient scavenging of lower concentrations of the radical by cellular antioxidant defences such as

superoxide dismutases and reducing species (glutathione *etc.*), rises in DEL only occurring when these defence systems are overwhelmed by very large concentrations of the chemical. Alternatively the lag may indicate clearing of lower concentrations of the chemical from the cell by non oxidative pathways.

The oxidation of aromatic amines, such as aniline, by oxidising agents and in air, occurs readily, to generate a radical cation. *In vivo*, resultant reduction by glutathione or other reducing species may lead to a radical cascade and, ultimately, to oxidative damage of DNA when defence systems are overwhelmed.

The toxicity of haloalkanes such as carbon tetrachloride, another Ames negative carcinogen detected by RS112, to liver cells is also thought to be due to involve free radical metabolites which may either have a prooxidant effect or result in haloalkylation of biomolecules (Poli *et al*, 1988).

The proposal of a role for free radical species in the induction of DEL by non-mutagenic carcinogens is, as yet, speculative, based on circumstantial evidence of similarities in dose response profiles between some oxidative mutagens and 'non-mutagenic' carcinogens, and the suggestion of the possible involvement of free radicals in the effects of some of the chemicals. Much further work is required in order to confirm or disprove this hypothesis.

A role for oxygen radicals in carcinogenesis is implied by a number of observations. As previously mentioned, the phorbol ester tumour promoting agents cause elevations in intracellular levels of oxygen radicals, and their effects can be mimicked by oxidative species such as benzoyl peroxide, or inhibited by scavengers of radical species (Marx, 1983). A role for

oxidative stress in the progression of naturally occurring tumours is implicit in the observation of a concomitant decrease in levels of the protective superoxide dismutase enzyme with decreasing levels of tumour cell differentiation and increased growth rate of the tumour. Additionally evidence of oxidative damage to these cells is more marked with tumour progression (Bartoli *et al*, 1982).

7.4.3. Proposals for Further Research.

So far only a very small range of oxidative chemicals have been studied for effects on recombination in RS112, an expansion of this range is obviously desirable. Chemicals should be chosen with reference to their possible modes of oxygen radical generation and the likely major radical product, *e.g.* production of O_2^- by redox active quinones. Similarly, the range of radical scavenging chemicals studied should be expanded and tested on other oxidative mutagens for any inhibition of recombinogenic effects. Other potential scavengers for study include mannitol and ascorbic acid.

It may be possible to measure the level of oxidative free radicals in cells directly *in vivo* by using the reporter compound 2',7'-dichlorodihydrofluorescein, which is oxidised by free radicals to the fluorescent 2',7'-dichlorofluorescein. The precursor molecule 2',7'-dichlorodihydrofluorescein diacetate is taken up into cells and trapped intracellularly by the action of a cellular esterase enzyme which removes the permeablising acetate moieties. It may therefore be possible to measure the levels of cellular oxidative stress in response to treatment with oxidative mutagens and non-mutagenic carcinogens, if not *in vivo*, then in cell extracts

made post-treatment.

Finally, the development of tester strains deficient in the protective enzymes superoxide dismutase and/or catalase may both increase the sensitivity of the tester strain towards oxidatively induced recombination and give more information as to the relative effects on DEL and ICR of O_2 generation (as generated by PQ, dismutated by SOD to generate H_2O_2 and molecular oxygen) and $OH^{\cdot}$ generation (from H_2O_2 which is removed by catalase to give water and molecular oxygen).

One simple, quick, initial experiment would be the investigation of the effect of DMSO on the induction of DEL by one of the non-mutagenic carcinogens, such as aniline. The observation of an attenuation of DEL induction by the radical scavenger would be strong evidence for the involvement of radical species in the induction. A negative effect need not rule out the involvement of radicals, however, as the effect of DMSO on the induction of recombination by H_2O_2 is undoubtedly due to the removal of $OH^{\cdot}$, which is probably not the only radical species capable of mediating damage to DNA.

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